

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :  G01N		A2	(11) International Publication Number: <b>WO 96/41144</b>																																																																								
			(43) International Publication Date: 19 December 1996 (19.12.96)																																																																								
(21) International Application Number: PCT/US96/08935  (22) International Filing Date: 4 June 1996 (04.06.96)		(81) Designated States: CA, CN, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).																																																																									
(30) Priority Data: 08/476,544 6 June 1995 (06.06.95) US		Published <i>Without international search report and to be republished upon receipt of that report.</i>																																																																									
(71) Applicant: HYPERION, INC. [US/US]; 14100 S.W. 136th Street, Miami, FL 33186 (US).																																																																											
(72) Inventors: DAN LIKER, Walter, Beach; 1024 Havenhurst Drive, La Jolla, CA 92037 (US). DEVLIN, Robert, Francis; 2240 Shelter Island Drive, San Diego, CA 92166 (US). ARRHENIUS, Peter, Olaf, Gustaf; 4304 Caminito del Zafiro, San Diego, CA 92121 (US). HSU, Mao-Lin; 18200 Devenwood Circle, Fountain Valley, CA 92708 (US).																																																																											
(74) Agents: BERKMAN, Charles, S. et al.; Lyon & Lyon, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).																																																																											
<p><b>(54) Title:</b> POLYOXYHYDROCARBYL RELATED PRODUCTS AND METHODS FOR FLUORESCENCE ASSAYS</p> <p><b>(57) Abstract</b></p> <p>Fluorescent dyes which are free of aggregation and serum binding are provided. These dyes are suitable for applications such as fluorescence immunoassays, <i>in vivo</i> imaging and <i>in vivo</i> tumor therapy. Fluorescence immunoassays methods are provided which use fluorescent dyes which are free of aggregation and serum binding. Such immunoassay methods are thus, particularly useful for the assay of biological fluids, such as serum, plasma, whole blood and urine. The present invention is directed to compositions comprising an oligonucleotide linked to a detectably labeled marker component comprising a fluorophore moiety which comprises a substantially planar, multidentate macrocyclic ligand coordinated to a central atom capable of coordinating with two axial ligands and two polyoxyhydrocarbyl moieties which are attached as axial ligands to the central atom. The present invention is also directed to nucleic acid hybridization and amplification methods employing such compositions.</p>																																																																											
<p><b>ANALYSIS: CHANNEL A</b></p> <table border="1"> <thead> <tr> <th>PEAK NO.</th> <th>TIME</th> <th>TYPE</th> <th>HEIGHT(μV)</th> <th>AREA(μV-sec)</th> <th>AREA %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>3.468</td> <td>N1</td> <td>3483</td> <td>22063</td> <td>0.061</td> </tr> <tr> <td>2</td> <td>4.051</td> <td>N2</td> <td>4.051</td> <td>2268025</td> <td>6.290</td> </tr> <tr> <td>3</td> <td>5.885</td> <td>N3</td> <td>58612</td> <td>11074450</td> <td>30.717</td> </tr> <tr> <td>4</td> <td>8.375</td> <td>N4</td> <td>110393</td> <td>313179</td> <td>0.868</td> </tr> <tr> <td>5</td> <td>11.071</td> <td>N5</td> <td>8152</td> <td>9470101</td> <td>26.267</td> </tr> <tr> <td>6</td> <td>13.351</td> <td>N6</td> <td>96740</td> <td>26815</td> <td>0.731</td> </tr> <tr> <td>7</td> <td>17.631</td> <td>N1</td> <td>5092</td> <td>1504825</td> <td>4.173</td> </tr> <tr> <td>8</td> <td>18.710</td> <td>N2</td> <td>17949</td> <td>1199819</td> <td>3.327</td> </tr> <tr> <td>9</td> <td>40.655</td> <td>N1</td> <td>76569</td> <td>6775683</td> <td>18.793</td> </tr> <tr> <td>10</td> <td>42.630</td> <td>N2</td> <td>38484</td> <td>3160570</td> <td>8.766</td> </tr> <tr> <td colspan="2">TOTAL AREA</td> <td></td> <td>36052530</td> <td>99.993</td> <td></td> </tr> </tbody> </table>				PEAK NO.	TIME	TYPE	HEIGHT(μV)	AREA(μV-sec)	AREA %	1	3.468	N1	3483	22063	0.061	2	4.051	N2	4.051	2268025	6.290	3	5.885	N3	58612	11074450	30.717	4	8.375	N4	110393	313179	0.868	5	11.071	N5	8152	9470101	26.267	6	13.351	N6	96740	26815	0.731	7	17.631	N1	5092	1504825	4.173	8	18.710	N2	17949	1199819	3.327	9	40.655	N1	76569	6775683	18.793	10	42.630	N2	38484	3160570	8.766	TOTAL AREA			36052530	99.993	
PEAK NO.	TIME	TYPE	HEIGHT(μV)	AREA(μV-sec)	AREA %																																																																						
1	3.468	N1	3483	22063	0.061																																																																						
2	4.051	N2	4.051	2268025	6.290																																																																						
3	5.885	N3	58612	11074450	30.717																																																																						
4	8.375	N4	110393	313179	0.868																																																																						
5	11.071	N5	8152	9470101	26.267																																																																						
6	13.351	N6	96740	26815	0.731																																																																						
7	17.631	N1	5092	1504825	4.173																																																																						
8	18.710	N2	17949	1199819	3.327																																																																						
9	40.655	N1	76569	6775683	18.793																																																																						
10	42.630	N2	38484	3160570	8.766																																																																						
TOTAL AREA			36052530	99.993																																																																							

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

DESCRIPTION

Polycxyhydrocarbyl Related Products And Methods  
For Fluorescence Assays

5

Field Of The Invention

The present invention relates to the field of assays, in particular fluorescence immunoassays, receptor assays, and hybridization assays.

10

Background Of The Invention

The determination of the presence or amount of substances is commonly performed by immunoassay. Immunoassay techniques are based on the binding of the antigenic substance being assayed (the "target analyte") to a receptor for the target analyte. The term "analyte" or "target analyte" refers to the compound to be measured in an assay which may be any compound for which a receptor naturally exists or can be prepared which is mono- or polyepitopic, antigenic or haptenic, a single or plurality of compounds which share at least one common epitopic site or a receptor. Immunoassay methods can be applied to the assay of many different biologically active substances. Among such substances are haptens, hormones, gamma globulin, allergens, viruses, virus subunits, bacteria, toxins such as those associated with tetanus and animal venom, and many drugs. Similar techniques can be used in non-immunological systems with, for example, specific binding proteins.

25

Many different types of immunoassays are known in the art, including competitive inhibition assays, sequential addition assays, direct "sandwich" assays, radioallergosorbent assays, radioimmunosorbent assays and enzyme-linked immunosorbent assays. The basic reaction underlying most immunoassays is the binding of certain substance, termed the "ligand" or "analyte", by a characteristic protein (receptor) to form a macromolecular

complex. These binding processes are reversible reactions, and the extent of complex formation for particular analyte and receptor concentrations is regulated by an equilibrium constant according to the law of mass action. Thus, at equilibrium, some of the analyte always exists unbound (free).

The measurement of target analytes in biological fluids, such as serum, plasma and whole blood, requires immunoassay methods which are both specific and sensitive. Both the specificity and sensitivity of an immunoassay depend on the characteristics of the binding interaction between the target analyte and the receptor involved. For example, the reaction must be specific for the analyte to be measured and the receptor used should not bind to any other structurally related compounds. In addition, by choosing a receptor with a high affinity for the target analyte, the sensitivity can be increased. The label used to monitor the assay also affects the sensitivity of an immunoassay. Either the target analyte or the receptor may be labeled to permit detection. Labels currently used for immunoassay of target analytes in biological fluids include radioisotopes (radioimmunoassay, RIA), enzymes (enzyme immunoassay, EIA), fluorescent labels (fluorescence immunoassay, FIA), and chemiluminescent labels (chemiluminescent immunoassay, CIA). Theoretically, fluorometry is capable of being one of the most sensitive of all analytic tools as it is possible to detect single photon events.

FIA's use fluorescent molecules as labels and may be either heterogeneous or homogeneous. Fluorescent molecules (fluorophores) are molecules which absorb light at one wavelength and emit light at another wavelength. See Burd, J.F., "Fluoroimmunoassay -- Application to Therapeutic Drug Measurement," in P. Moyer et al., Applied Therapeutic Drug Monitoring, American Association of Clinical Chemistry (1984). Typically, an excitation pulse of radiation is directed onto or into a sample, followed

by fluorescence of the sample, and the detection of the fluorescence radiation. Fluorescence is a phenomenon exhibited by certain substances, which causes them to emit light, usually in the visible range, when radiated by another light source. This is not reflection, but creation of new light. Current commercially available assay methods use fluorescein, which emits green light when radiated by a light source containing blue light. In addition to fluorescing, fluorescein (and other fluorophores) emit polarized light. That is, the light emitted has the same direction of polarization as the incident polarized light, if the fluorescein molecule is held fixed with its transition moment parallel to the electric field of the excitation.

Using a fluorescent polarizing probe in a competitive binding immunoassay provides a type of FIA called a fluorescence polarization immunoassay (FPIA). In this type of assay, the smaller the molecule is, the smaller its rotational relaxation time and the faster it rotates. Typically, antibody molecules are much larger than drug or drug-probe molecules. One advantage of the polarization technique is the elimination of a step to separate unbound probe. Although the unbound tracer is not physically eliminated from the samples in FPIA, its contribution is readily assessed by the polarization. Another advantage in the FPIA technique is lack of dependence on intensity. Unlike most assays using a light measurement, in which it is the intensity of the light that is correlated to drug concentration (so any variations in source light intensity will directly affect the sensitivity of the assay), the sensitivity of FPIAs is independent of intensity variations. Conventional FPIAs require separate measurements of both blank and sample.

One problem which has plagued fluorescence immunoassays has been discriminating the fluorescent signal of interest from background radiation. The intensity of signal from background radiation may be up to

10,000 times larger than the intensity of the fluorescent signal of interest. The problem of background detection is particularly pronounced in assay of biological samples. Many of the current fluorescence assays use the 5 fluorescent molecule, fluorescein. Fluorescein has an excitation maximum of 493 nm, and there are numerous substances in biological fluids with overlapping excitation and emission similar to fluorescein. For example, in the analysis of blood plasma, the presence of 10 a naturally occurring fluorescable material, biliverdin, causes substantial background radiation. Such compounds are highly fluorescent and contribute significant background signals which interfere with the label's signal, thus limiting the sensitivity of assays using 15 fluorescein labels.

Two factors commonly viewed as going hand in hand and contributing to the problem background radiation are: (1) solvent sensitivity and (2) non-specific binding. Solvent sensitivity refers to tendency of a solvent to affect the 20 fluorescent signal of the dye. For example, several dyes that are fluorescent in organic solvents tends to aggregate in aqueous solutions and therefore exhibit quenched fluorescence. Non-specific binding refers to the tendency of sample components to interfere with the 25 fluorescent signal of the dye. For example, serum components such as HSA often bind to conventional fluorescent dyes and quench or enhance interfere with the fluorescent signal.

It has been recognized that for analysis of 30 biological fluids, it would be desirable to use a dye or label which is excitable at radiations of wavelengths of greater than background radiation. However, even though the background fluorescence of serum falls off at wavelengths approaching 600 nm, significant interference persists until 650 nm or greater. Previous attempts to 35 create dyes of such wavelengths have been unsuccessful. See, e.g., Rotenberg, H. and Margarfit, R., Biochem.

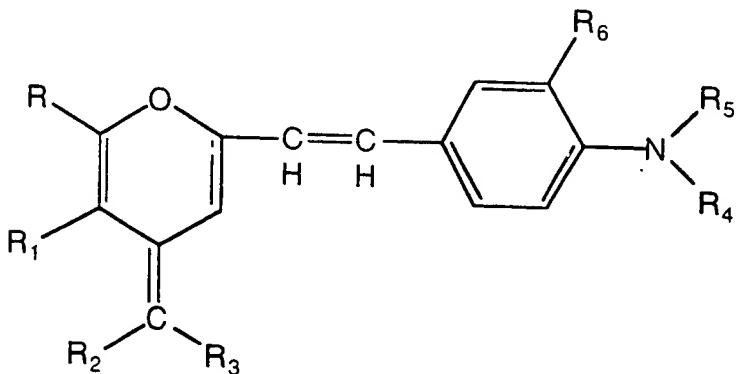
Journal 229:197 (1985); and D.J.R. Laurence, Biochem. Journal 51:168 (1952). Fluorescent dyes having emission wavelengths which reduce interference from background fluorescence include cyanines, porphyrins and azaporphyrins. However, it has been found that the use of such labels in fluorescence assays is limited by the problems of solvent sensitivity (significantly decreased fluorescence intensity in the aqueous assay solution in comparison to dimethylformamide) and non-specific binding to biological materials (significantly decreased fluorescence intensity in purified or isolated sample comparison to a sample containing serum components such as HSA).

Use of fluorophores having long decay times is especially important in techniques such as transient state assays where there is a need for fluorophores whose emissions may be measured over a time period of up to about 20 nanoseconds. It has been found that for fluorophores natural lifetime and extinction coefficient vary antitatively (*i.e.*, when one increases, the other decreases; although they need not change at rates inversely to each other). Also, fluorophores having longer fluorescent lifetimes are more apt to be deactivated. Accordingly, fluorophores having enhanced decay times, *i.e.* having decay times which approach their natural lifetime, offer greater quantum yields and, thus, greater sensitivity.

Earlier attempts to overcome the problem of background radiation have involved: (1) the use of filters to reject detected radiation at all but a narrowly defined wavelength band; (2) binding fluorescent probes to a macro-molecular support thereby minimizing the effect of serum proteins while allowing detection of the binding to a target analyte, (see U.S. Patent No. 4,615,986, issued October 7, 1986); and (3) the use of chemical groups designed to improve the functional properties of the dyes

(see Mujumdar, et al. Bioconjugate Chemistry 4: 105-110, 1993).

5 Arnost et al., U.S. Patent No. 4,886,744, issued December 12, 1989 ("Arnost") describes a fluorescent label with an unsymmetrical cyanine dye of structure:



Formula I

10 Certain of the R groups can be "a hydrophilic group". Hydrophilic groups are defined as including a variety of possibilities, including a polyether, in order to decrease aggregation and non-specific binding. The data set forth by Arnost are said to show that some of the 15 "hydrophilic groups" decrease the nonspecific binding somewhat and lowered the binding constants from 7E5 down to 1.5E4, a factor of less than 50. The components of Arnost, et al. typically have an excitation maximum of less than 500 nm.

20

#### Summary Of The Invention

The present invention relates to marker components, 25 fluorescent probes, oligonucleotides, hybridization assays, and immunoassays using such products and methods for making such products. According to the present invention, detectably labeled marker components are provided that comprise a fluorophore moiety coupled to one or more polyoxyhydrocarbyl moieties. The polyoxyhydrocarbyl moieties preferably reduce or remove

the problems of solvent sensitivity and non-specific binding.

5        Use of such detectable labels or marker components in immunoassays is advantageous in that these labels have substantially the same intensities of parallel and perpendicular components of transient state fluorescence emission in the presence and absence of biological fluids such as serum. Thus, assay methods using these labels are capable of detecting low concentrations of target analyte  
10      in biological fluids.

These marker components may be used as labels for labeling an analyte, antigen, antibody or other molecule. These marker components may be optionally functionalized so as to include a linker arm which allows the marker component to be linked to the analyte, antigen, antibody or other molecule. A variety of linker arms which are suited to this purpose have been described. Kricka, J.J.;  
15      Ligand-Binder Assays; Labels and Analytical Strategies; pages 15-51; Marcel Dekker, Inc., New York, NY (1985).  
20      The marker component is linked to the analyte, antigen, antibody or other molecule using conventional techniques.

In one aspect the present invention provides a detectably labeled marker component which comprises: (1) a fluorophore moiety comprising a luminescent substantially planar molecular structure having excitation wavelengths of at least about 500 nm and (2) coupled thereto one or more polyoxyhydrocarbyl moieties. Examples of preferred fluorophores, polyoxyhydrocarbyl moieties, and linkages of the two are described in detail herein. In addition,  
25      evidence is provided demonstrating the effectiveness of the marker components at reducing solvent sensitivity and non-specific binding.

30      In especially preferred embodiments, the marker components of the present invention can be used to make probes as generally described in commonly owned U.S. application Serial No. 08/051,446, filed April 21, 1993, and used in immunoassays as generally described in  
35

commonly owned U.S. application Serial No. 08/035,633, filed March 23, 1993, the disclosure of both of which are incorporated herein by reference in their entirety, including any drawings.

5

Brief Description Of The Drawings

Figure 1 is a RPHPLC elution diagram of a reaction carried out in 0.1M, pH 9.3 carbonate buffer between bis-reactive CY5.5 and PEG-amine. Non-specific binding was absent in Peaks 9 and 10.

Figure 2 is similar to Figure 1 except that the reaction was carried out under anhydrous conditions in a mixture of DMF and NMP. Peaks a, b and c all showed strong non-specific binding whereas non-specific binding was absent for peaks 1 and 2.

Figure 3 is a RPHPLC elution diagram of the same reaction mixture as in Figure 2 except after removal of DMF and NMP by rotary evaporation and vacuum drying.

Figure 4 is a RPHPLC elution diagram of a fast moving peak in the separation on Biogel P-6.

Figure 5 is similar to Figure 4 except for the slow moving peak from P-6 separation.

Figure 6 shows the behavior of the RPHPLC fractions from reaction mixture 199-146A (reaction in DMF+NMP) when tested with a panel of sera or serum components to reveal non-specific binding. Polarization of the transient state fluorescence served as the index of non-specific binding. As a reference, data for an especially preferred dye of the present invention termed La Jolla Blue are also shown. The first bar in each group gives the polarization of the dye or fraction in question as observed in buffer alone (TD<sub>x</sub> buffer). The data shows that Peaks A, B and C (corresponding to a, b, and c, respectively, in Figure 2) bind strongly when run with the test panel while La Jolla Blue, Peak 1 (Fig.2), and Peak 2 (Fig.2) show negligible binding. The differences between the polarizations of La Jolla Blue (197 239B), Peak 1 and Peak 2 in buffer alone

are a reflection only of the differences in the photophysical properties of the different dyes structures and are not associated with aggregation or the presence or absence of ncn-specific binding. Thus it can be seen that 5 CY 5.5 after reaction with PEG-amine is virtually free of non-specific binding and that Peaks a, b and c correspond to various forms of bare, unprotected dye while Peaks 1 and 2 are coupled to PEG.

Figure 7 shows addition 1 examples of dyes that can 10 be used in the present invention. The compound numbers correspond to those used in Organic Colorants, Okawara, et al., 1986, incorporated herein by reference in its entirety including any drawings.

15 Detailed Description Of The Invention

The following description includes preferred modes of carrying out the invention and is made for the purpose illustrating the general principles of the invention rather than limiting the invention in any manner.

20

I. PREFERRED MARKER COMPONENTS

A. Preferred Fluorophore Moieties

Accordingly, in general, preferred are fluorophores which efficiently produce fluorescence upon excitation 25 with light whose wavelength falls in the range of about 200 to about 1000 nanometers, preferably in the range of about 600 to 800 nanometers more preferably the range above 650 nm. Suitable fluorophores include those which absorb and/or emit at wavelengths which are 30 distinguishable from the excitation and emission maxima of other solution components (such as proteins present in a sample) to minimize background fluorescence.

Since these marker components are particularly useful 35 in assays using samples of biological fluids, for those uses, preferred are fluorophores having excitation and/or emission wavelengths of at least about 500 nanometers which reduces interference from the ambient fluorescence

of other sample components. Some samples, such as serum, may exhibit considerable interfering background fluorescence from flavins, flavoproteins, NADH, etc. when excitation wavelengths less than 500 nm are used.

5 For certain applications, such as fluorescence polarization immunoassays, preferred fluorophores may also exhibit a high degree of fluorescence polarization when bound and a low degree of polarization when bound. For certain applications such as fluorescence transient state assays, preferred fluorophores are also characterized by measured fluorescence decay times in the range of about 1 nanosecond to about 50 nanoseconds, preferably in the range of about 5 to about 20 nanoseconds. For other applications, such as phosphorescent labels, fluorophores having even longer decay times may be used. The term "decay time" is the time which must elapse in order for the concentration of excited molecules to decrease from its initial concentration to  $1/e$  of that value.

20 Thus, preferred are fluorophores which produce fluorescent light efficiently, i.e., which are characterized by high absorbtivity at the appropriate wavelength and high fluorescence quantum yields. For certain applications, preferred fluorophores have measured fluorescence decay times on the order of at least 2 nanoseconds and exhibit a high degree of fluorescence polarization.

25 Suitable fluorophore moieties comprise a luminescent substantially planar molecular structure. One class are fluorophore moieties in which the luminescent substantially planar molecular structure comprises a substantially planar macrocyclic multidentate ligand which coordinates a central atom which may coordinate with two axial ligands, one on either side of the macrocyclic ligand (i.e. having a trans orientation). The term "axial ligand" refers to a substituent which, together with a macrocyclic ligand, forms a coordination complex with a

central atom. The axial ligand lies normal to the plane described by the macrocyclic ligand.

Preferred central atoms are elements which may form octahedral coordination complexes containing two ligands with a trans or axial orientation, on either side and perpendicular to the planar macrocyclic ligand. For use as fluorescent marker components in certain applications the central atom should not have too high atomic number (about 30 or less) so that fluorescence is largely lost by transition to the triplet state. For uses such as in vivo tumor therapy, higher atomic weight atoms may be used, or in separation-type assays or for phosphorescent labels. For use as marker components in fluorescence immunoassays, suitable central atoms are those to which may coordinate two axial ligands and which are not of high enough atomic number to cause extensive fluorescence quenching by transition to the triplet state. Preferred elements for the central atom include silicon, germanium, phosphorus, and tin, especially preferred are silicon and germanium.

Preferred multidentate ligands include nitrogen-containing macrocycles which have conjugated ring systems with pi-electrons. These macrocycles may be optionally substituted, including substitution on bridging carbons or on nitrogens. Suitable macrocycles include derivatives of porphyrins, azaporphyrins, corrins, sapphyrins and porphycenes and other like macrocycles having conjugated pi-electron ring systems. In view of the fact that they incorporate many of the above-noted characteristics, an especially preferred class of macrocycles comprise porphyrin derivatives, and azaporphyrin derivatives (porphyrin derivatives wherein at least one of the bridging carbons is replaced by a nitrogen atom). Azaporphyrin derivatives include derivatives of mono-, di- and triazaporphyrin and porphrazine. These macrocycles may optionally have fused aromatic rings. These azaporphyrin derivatives include phthalocyanine, benzotriazaporphyrin and naphthalocyanine and their

derivatives. The preparation and fluorescent qualities of many of these compounds are known and some are available commercially. Moser, F.; Phthalocyanine Compounds; Reinhold Publishing Co., New York (1963); Wilkinson, G. (editor); Comprehensive Coordination Chemistry; Volume 2, pages 813-898; Pergamon Press, New York (1987); Hanack, M., et al.; "Synthesis and Properties of a New Kind of One-Dimensional Conductor", Journal of Organometallic Chemistry 204:315-325 (1981); and Lezhoff, C.C. and Lever, A.S.P. (editors); Phthalocyanines: Properties and Applications; VCH Publishers, Inc., New York (1989).

Other classes or fluorophores to which one or more polyoxyhydrocarbyl groups may be attached according to this invention are indicated in the examples and figure 7 below.

For certain applications, such as fluorescence polarization assays, preferred macrocycles are azaporphyrin derivatives which exhibit a high degree of polarization, that is, those which emit strongly polarized light. For these applications, preferred are macrocycles having lower degrees of symmetry, preferably having lower symmetry than  $D_{4h}$ . One preferred group includes macrocycles having at least one fused aromatic ring or peripheral substituent. Thus, preferred macrocycles include azaporphyrin derivatives having fused aromatic rings at positions which result in decreased symmetry. Preferred classes of azaporphyrin derivatives comprise derivatives of monoazaporphyrin, diazaporphyrin, and triazaporphyrin having lower than  $D_{4h}$  symmetry.

Preferred fluorophores include: (1) polymethine dyes; (2) quinoid dyes; (3) indanthrene dyes; (4) indigoid dyes; (5) azines, such as Nile Blue A and laser dyes; (6) arylmethane dyes and heterocyclic analogs; (7) pyrylium, thiopyrylium, and squarylium dyes; (8) quinone methides; (9) conjugated betaine dyes (derivatives of pyridinium N-cyclopentadienide); and (10) macrocycles such as porphyrins, hydroporphyrins, azaporphyrins including

phthalocyanines, corroles, corrins and pentapyrrole macrocycles such as sapphyrins. Examples of such dyes are provided below.

5 Polymethine dyes include symmetrical and unsymmetrical cyanines either of which can be cationic, anionic or neutral cyanines (merocyanines). The structures of the groups terminating the polymethine chain determines into which of the above classes a particular cyanine falls.

10 The longest wavelength of absorption is determined by both the terminal groups and the number of -CH=CH- groups in the polymethine chain. Substitution along the polymethine chain can produce either red shifts or blue shifts depending upon the 15 structure of the substituent group and its position in the chain.

20 Other structural modifications can be made by cyclization or by coupling structures together. Hence, it can be appreciated that the number of cyanines possible is very large. Thousands of these compounds have been synthesized in the search for better photographic sensitizers.

25 Especially preferred fluorophores include: (1) aryl terminated polymethine dyes, such as trimethines, pentamethines, and heptamethines; (2) quinoid dyes such as 1-Amino-2-benzoyl-4-(arylarnino)anthraquinones, 6-(Arylamino)naph[2,3,c]acridan-5,8,14-triones; (3) indanthrene dyes; (4) 1,4-diaminoanthraquinone-2,3-dicarboxamides; (5) tetraaminoanthraquinones; (6) azine dyes; (7) pyrylium, thiopyrylium and squarylium dyes; and (8) naphthoquinone methides.

#### B. Preferred Polyoxvhydrocarbyl Moieties

35 Preferred solubilizing polyoxyhydrocarbyl moieties include water soluble carbohydrates such as glucose, sucrose, maltotriose and the like; water soluble carbohydrate derivatives such as gluconic acid and

mannitol, and oligosaccharides; polypeptides such as polysine and naturally occurring proteins; and water soluble polymers such as polyvinylpyrrolidone, poly(vinyl alcohol), poly(ethylenimine), polyacrylic acid, 5 polyacrylamide, ethylene oxide copolymers such as Pluronic™ (a polyether) and Tetronic™ (BASF) polyol surfactants and, in particular, polyethers such as other polyoxyalkylenes including poly(ethylene glycol), or other water soluble mixed oxyalylene polymers, and the like.

10 A particularly preferred class of solubilizing polyoxyhydrocarbyl moieties comprises poly(ethylene glycol) (PEG) and poly(ethylene glycol) derivatives, such as poly(ethylene glycol) monomethyl ether. Other suitable PEG derivatives include PEG-silicon derived ethers. Many 15 of these polymers are commercially available in a variety of molecular weights. Others may be conveniently prepared from commercially available materials, such as by coupling of an amino-PEG to a haloalkyl silyl or silane moiety. When linked to a fluorophore moiety, these 20 polyoxyhydrocarbyl moieties impart particularly advantageous qualities of solubility in aqueous solution with improved measured fluorescence decay time, and improved fluorescence intensity. Moreover, the resulting marker components are water soluble and show decreased 25 non-specific binding, especially decreased binding to serum albumin which has heretofore been a problem with certain fluorophores, particularly porphyrin or phthalocyanine dyes which have been functionalized with groups such as sulfonate to impart increased water solubility to the molecule. Non-specific binding of 30 fluorophore or marker component impairs the accuracy of the resulting immunoassay. These marker components which comprise fluorophore linked to PEG may also exhibit improved fluorescence intensity in aqueous solution with decreased quenching.

35 Suitable PEGs may vary in molecular weight from about 200 to about 20,000 or more, more preferably 2,000 to

20,000, more preferably 4,000 to 15,000, most preferably 8,000 to 10,000. Choice of a particular molecular weight may depend on the particular fluorophore chosen and its molecular weight and degree of hydrophobicity, as well as 5 the particular application for which the fluorophore-PEG complex is to be used.

C. Absorbance and Polarization Behavior of Preferred Marker Components

10 The lack of solvent sensitivity and non-specific binding to HSA and serum components was demonstrated by measurement of absorbance spectra and transient state fluorescence emission. Marker components which comprise a central atom (for example, silicon) coupled to one or more 15 polyoxyhydrocarbyl moieties such as PEG may be sensitively characterized by measurements of transient state fluorescence. In such measurements the intensity of the two components polarized either parallel or perpendicular to the direction of polarization of the exciting pulse is 20 monitored over a time period equal to about 3 times the decay time of the marker component. Such curves reflect extinction coefficient, quantum yield, decay time and state of polarization and supply sensitive indications on the chemical and physical condition of the marker 25 component.

For example, if the excited state is being deactivated or converted to the triplet state the overall intensities are lowered and the decay times shortened. If 30 the rotary brownian motion of the molecule is being altered by an increase in viscosity or by being bound to a large molecule, the ratio of the intensity of the parallel to the perpendicular component is increased. The term "bound" refers to the condition in which a binding interaction has been formed between a molecule and its 35 specific binding partner.

Some marker components according to the present invention show, within experimental error of about 5%, the

same intensities, decay time and polarization in DMF (an organic solvent) as in SAP (saline azide phosphate, an aqueous neutral buffer). To some extent these properties are shared by other marker component preparations. A 5 distinctive and important property of the marker components of the present invention is an insensitivity to (and lack of binding to) the components in serum which is evidenced by a lack of any measured effect of serum on the 10 intensities, decay time or relative magnitudes of the polarized components of the fluorescence. This property is crucial for the marker components to be useful for applications such as assays using biological materials.

The absorbance spectra in the visible and near-infrared range for a product of hydroxy silicon 15 phthalocyanine with PEG monomethyl ether of average molecular weight 350 show that the positions and heights of the absorbance maxima are nearly identical in DMF, an organic solvent and in SAP (saline azide phosphate), an aqueous buffer solution. In contrast, silicon 20 phthalocyanine which is unsubstituted with the solubilizing polyoxyhydrocarbyl axial ligands would be nearly insoluble in either of these solvents and would exhibit only very low absorbance levels in DMF and virtually none in SAP.

25 Transient state fluorescence emission for the same PEG-substituted silicon phthalocyanine as used above show that emission was virtually unaffected by the addition of human serum to sample in SAP. A silicon phthalocyanine solubilized by derivatization of the phthalocyanine 30 macrocycle with sulfonate without the PEG ligands would show changes in both fluorescence intensity and polarization when serum was added. Those changes are abrogated by the replacement of the hydroxy groups with the PEG axial ligands.

35 Transient state fluorescence emission for a PEG-substituted silicon phthalocyanine where the phthalocyanine macrocycle is sulfonated demonstrated the

same transient state emission in the presence of HSA, and HSA plus serum, as it did in buffer alone (SAP). Sulfonation of the macrocycle did not affect the ability of the axial polyoxyhydrocarbyl solubilizing moieties to prevent nonspecific binding of the marker component to either HSA or serum components.

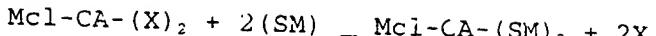
Transient state fluorescence emission for a sulfonated silicon phthalocyanine which does not have any solubilizing polyoxyhydrocarbyl moieties linked to the central silicon atom in DMF shows the two components polarized either parallel or perpendicular with respect to the polarization of the excitation flash to be about equal. Transient state fluorescence of the same material in SAP at the same concentration shows that the material is solvent sensitive and that its fluorescence is about 40% quenched in SAP. The addition of serum to the solution produced an enhancement of fluorescence and induced polarization of the emission of this dye such that the parallel and perpendicular components were not of substantially equal intensity. This indicates substantial binding of the sulfonated silicon phthalocyanine to serum components. The change in intensity after addition of the serum is also indicative of binding to serum components.

Visual and near-infrared absorbance of a sulfonated silicon phthalocyanine in DMF show wavelength maxima and absorbances are 673 nm (0.634) and 603 nm (0.107) respectively. The absorption spectrum of the sulfonated silicon phthalocyanine in SAP at the same concentration show wavelength maxima and absorbance are 678 nm (0.509) and 606 nm (0.092), respectively.

## II. Preparation Of Preferred Marker Components

According to one method of preparing the preferred marker components of the present invention, the appropriate fluorophore moiety having hydroxy or halide groups as axial ligands is reacted with a reactive form of the solubilizing polyoxyhydrocarbyl moiety in a ligand

exchange reaction according to the general reaction scheme:



wherein Mcl denotes the macrocyclic ligand, CA the central atom, X the displaced ligand and SM the solubilizing moiety. This reaction may be carried out neat or, if desired, in solvent. Suitable solvents include quinoline, THF, DMF, imidazole and the like. Suitable reaction temperatures may vary, depending on the nature of the macrocyclic starting material and the solubilizing group. The reaction is generally complete in about 2 minutes to about 24 hours. The reaction mixture can be conveniently heated under reflux or by means such as a sand bath. For convenience, the reaction may be carried out at ambient pressure.

It is believed that this reaction takes place in two steps, with one polyoxyhydrocarbyl group coordinating as an axial ligand at a time. The reaction of the polyoxyhydrocarbyl moiety with the central atom coordinated macrocyclic ligand may proceed in stages. Products of the reaction of PEG with dihydroxy silicon phthalocyanine ( $\text{PcSi(OH)}_2$ ) were observed. At the first stage ("Early Blue Stage"), the fluorophore moiety ( $\text{SiPc}$ ), while being rendered soluble in both DMF and SAP (aqueous saline azide phosphate buffer), was markedly sensitive to solvent and was about 85% quenched. Product of the latter stage of the reaction ("Blue Green Product"), in contrast, was totally insensitive to solvent and showed the same emission intensity and decay time in either solvent.

Preferred synthetic methods are provided in example 5 of commonly owned U.S. Patent 5,403,928, issued April 4, 1995.

### III. Utility

The marker components of the present invention are useful as fluorescent labels for fluorescent probes and in fluorescence immunoassays and also in as labels for in

vivo imaging and in vivo tumor therapy. These marker components may be advantageously used as fluorescent labels in conventional fluorescence immunoassays, including fluorescence polarization immunoassays. When so used, these marker components may be linked to one member of a specific binding pair ("labeled binding partner") or an analog of such a member. The marker component may be directly attached or conjugated thereto or attached or conjugated via a linker a. n. The term "specific binding pair" refers to two different molecules (or compositions) wherein one of the molecules has an area on the surface or in a cavity which specifically recognizes and binds to a particular spatial and polar organization of the other molecule or molecular complex involving other molecules. The term "binding partner" refers to a molecule or molecular complex which is capable or specifically recognizing or being recognized by a particular molecule or molecular complex.

These labeled binding partners are useful in assays having a variety of formats, such as assays which involve competition for analyte or analyte binding partner (if a labeled analyte or analyte-analog as used) and may be used in either homogeneous or heterogeneous assays. In view of their advantageous freedom from aggregation in aqueous solution and lack of solvent sensitivity (indicating no detectable aggregation) in combination with their lack of nonspecific binding to serum components and other biological macromolecules, these markers are especially suited for use in assays for detecting an analyte in a sample containing a biological fluid such as serum. Thus, these marker components may be used as labels for fluorescent probes for detecting analytes in solutions where non-specific binding by serum components would severely compromise sensitivity of an assay, affecting both its accuracy and precision.

Alternatively, these marker components may be used as agents for in vivo imaging. When used as imaging agents,

these marker components are conjugated to one member of a specific binding pair to give a labeled binding partner. The labeled binding partner is introduced into an animal. If the other member of the specific binding pair is present, the labeled binding partner will bind thereto and the signal produced by the marker component may be measured and its localization identified.

These marker components may also be used in in vivo tumor therapy. For example, photodynamic therapy involves using the marker component as a photosensitizing agent. The marker component (fluorescent label) is conjugated to a binding partner which may specifically recognize and bind to a component of a tumor cell. The localized triplet emission from the bound marker component conjugate after excitation by light, causes chemical reactions and selective damage and/or destruction to the tumor cells.

To assist in understanding the present invention, the following examples are included which describe the results of a series of experiments. The following examples relating to this invention should not, of course, be construed in specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall within the scope of the invention as described herein and herein after claimed.

Example:

Dyes of the phthalocyanine, the porphyrin and the cyanine or polymethine classes have been coupled to one or more polyoxyhydrocarbyl groups, e.g., PEG (polyethylene glycol). In every case the presence of the polyoxyhydrocarbyl group(s) resulted in a dramatic decrease in the tendency to aggregate and even more importantly in the tendency to bind non-specifically to biological materials, (human serum or human serum components in the actual experiments).

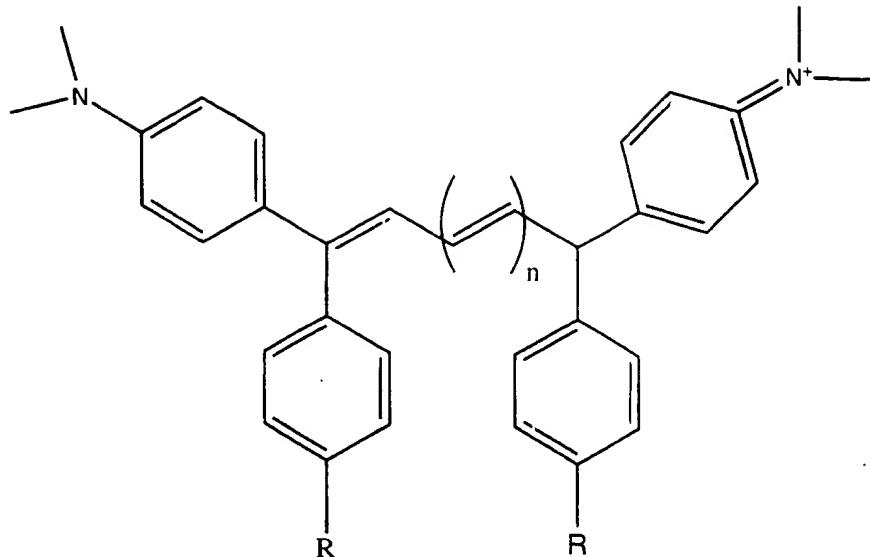
We have compared bare unprotected dyes to those coupled to polyoxyhydrocarbyl groups in the mix-and-read assay for digoxin in the presence of serum or whole blood. In this assay protection of the fluorescent label (a phthalocyanine) is absolutely essential for achieving the sensitivity needed to monitor digoxin levels in patients under digoxin therapy. A probe made with a bare phthalocyanine would be useless for any practical application. The digoxin assay which is presently dominant in the therapeutic drug assay market utilizes a bare dye (fluorescein) in the probe. However, in order to perform the assay a separate, off-line precipitation and centrifugation must first be done to denature and remove serum constituents which would otherwise make the assay impossible.

We have outlined methods for engineering diverse classes of IR / near IR dyes which have photophysical parameters suggesting their usefulness as fluorescent probes. The reactions shown below indicate how various dyes could be engineered to eliminate aggregation and nonspecific binding. Similar schemes can be written to convert such engineered dyes into useful probes by attachment of the appropriate hapten, antibody or other biomolecule.

25

Example 1: Aryl-terminated polymethine dyes

Aryl-terminated polymethine dyes that can be coupled to one or more polyoxyhydrocarbyl moieties include those described in W. B. Tuemmler, B. S. Wildi, J. Org. Chem. 1958, 80, 3772-3777; R. Wizinger, G. Renckhoff, Helv. Chim. Acta 1941, 24, 369E-388E; and H. Lorenz, R. Wizinger, Helv. Chim. Acta 1945, 28, 600-612, all of which are incorporated herein by reference in their entirety, including any drawings. Aryl-terminated polymethine dyes that can be coupled to one or more polyoxyhydrocarbyl moieties are preferably of the structure:



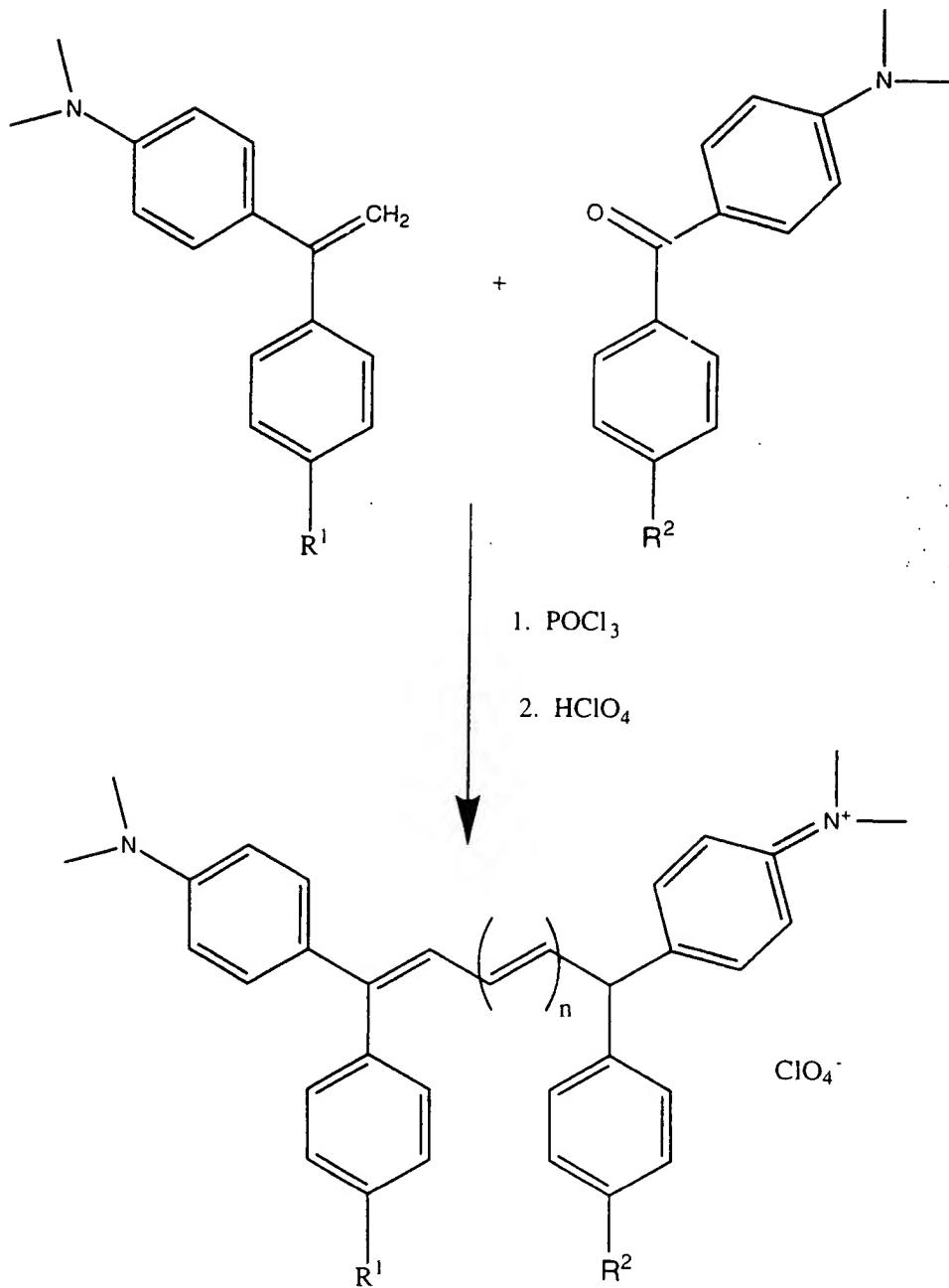
wherein n is 0, 1, or 2 and R is H, Cl, or N(CH<sub>3</sub>)<sub>2</sub>.

5

Aryl-terminated polymethine dyes of the above structure have the following spectral properties:

R	n	$\lambda_{\text{max}}$ (nm)	$\epsilon$
H	0	755	100,000
H	1	833	208,000
H	2	935	195,000
Cl	0	770	100,000
Cl	1	845	187,000
Cl	2	942	151,000
N(CH <sub>3</sub> ) <sub>2</sub>	0	740	36,000
N(CH <sub>3</sub> ) <sub>2</sub>	1	809	183,000
N(CH <sub>3</sub> ) <sub>2</sub>	2	911	186,000

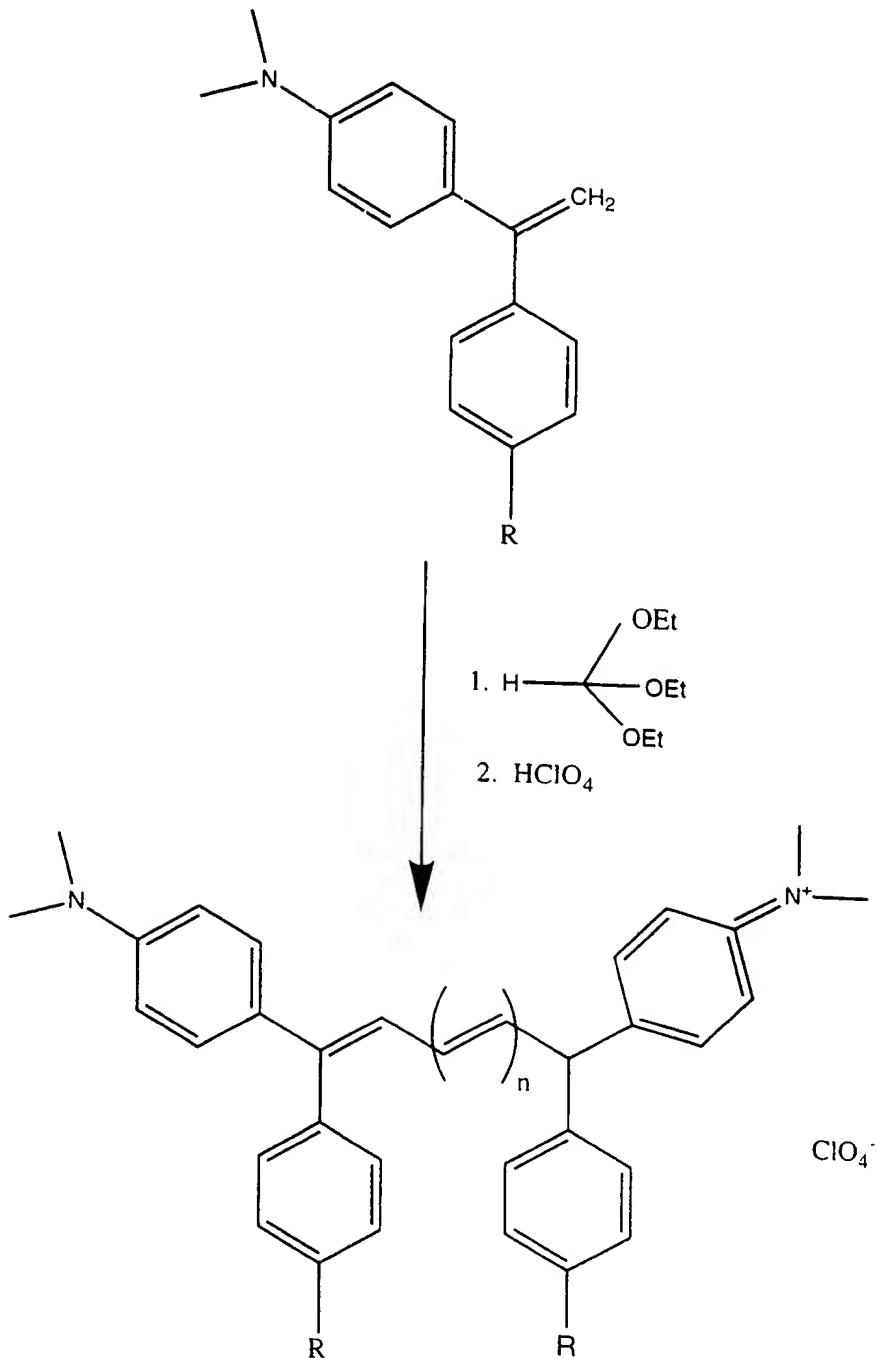
The aryl-terminated polymethine dyes of the above structure can be synthesized as described in W. B Tuemmller, S. S. Wildi *J. Org. Chem.* 1958, 23, 3772-3777. For example, trimethines (n = 0) can be synthesized as follows:



wherein 39-56% yields are obtained if  $n$  is 0 and  $R_1$  and  $R_2$  are H, Cl, or  $N(CH_3)_2$ .

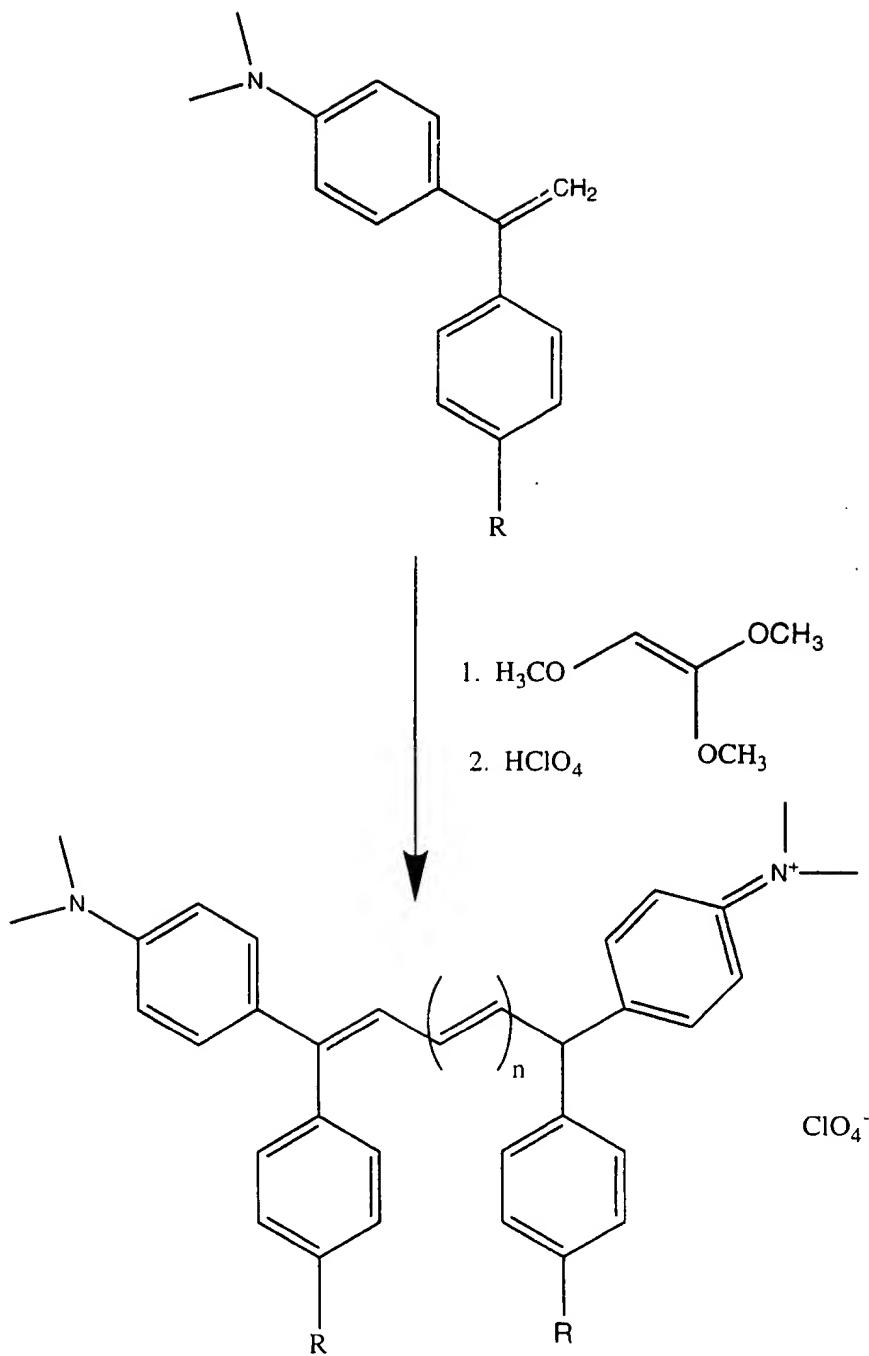
5      Similarly, pentamethines ( $n = 1$ ) can be synthesized as follows:

24



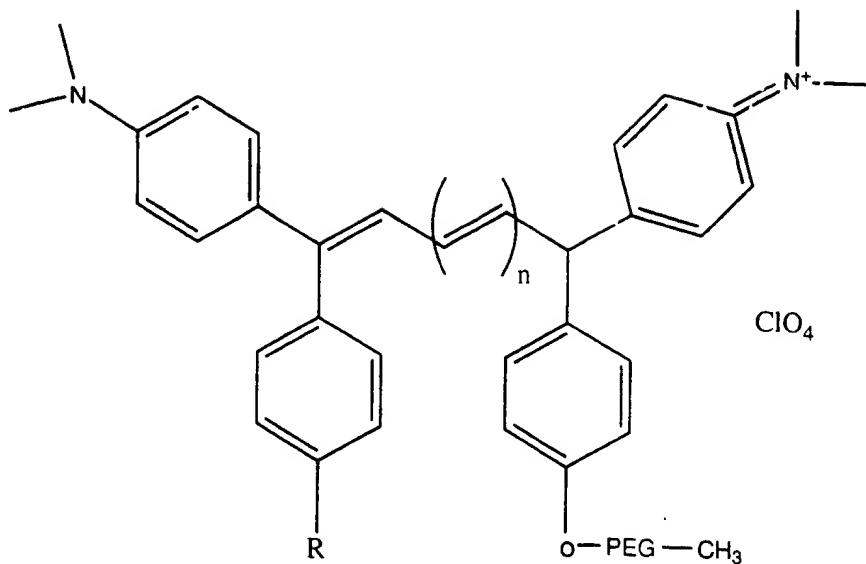
wherein yields of 50-99% may be obtained if  $n$  is 1 and R is H, Cl, or  $\text{N}(\text{CH}_3)_2$ .

25



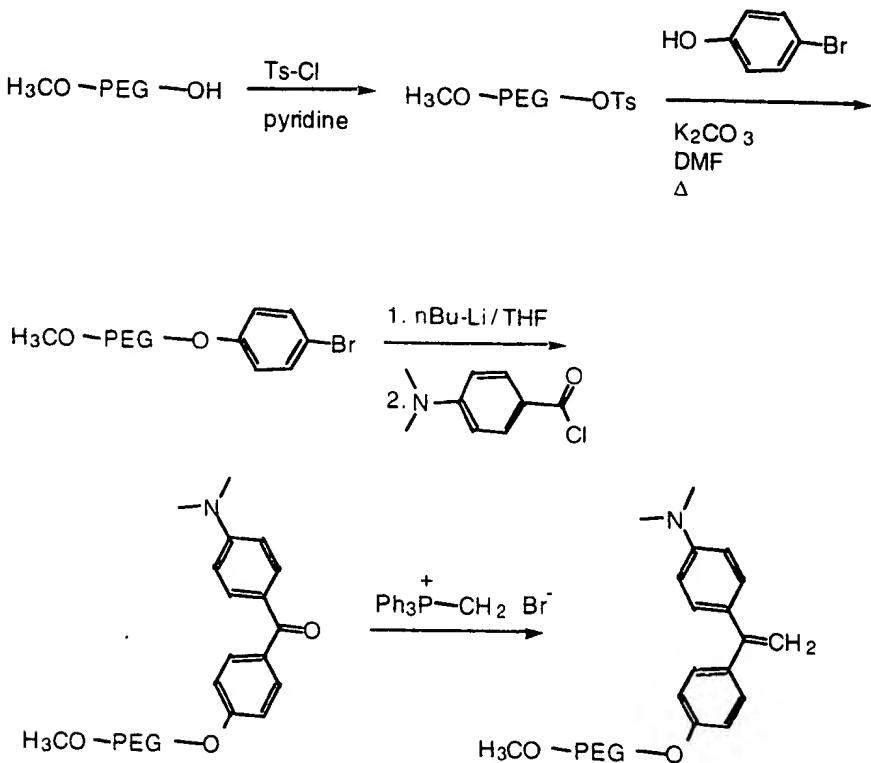
wherein yields of 37-96% can be obtained if n is 2 and R is H, Cl, or  $\text{N}(\text{CH}_3)_2$

5 Polyethylene glycol (PEG) conjugates of aryl-terminated polymethine dyes include:



wherein n is 0, 1, or 2 and R includes, but is not limited to H, Cl, Br, I, N(CH<sub>3</sub>)<sub>2</sub>, linker and O-PEG-OCH<sub>3</sub>.

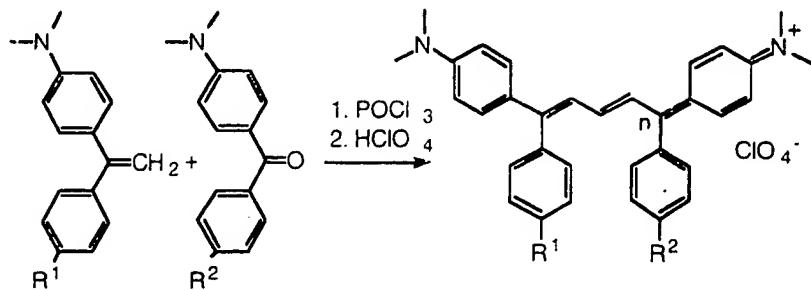
These PEG containing dyes can be synthesized as follows:



This product is the key intermediate for the synthesis of Teummller and Wildi given above and provides the polymethine dyes as follows:

5

For PEG Trimethines:



$n = 0, R^1 = H; R^2 = O\text{-PEG}\text{-OCH}_3$

$n = 0, R^1 = \text{Cl}, \text{Br}, \text{I}, \text{F}; R^2 = O\text{-PEG}\text{-OCH}_3$

$n = 0, R^1 = \text{linker group}; R^2 = O\text{-PEG}\text{-OCH}_3$

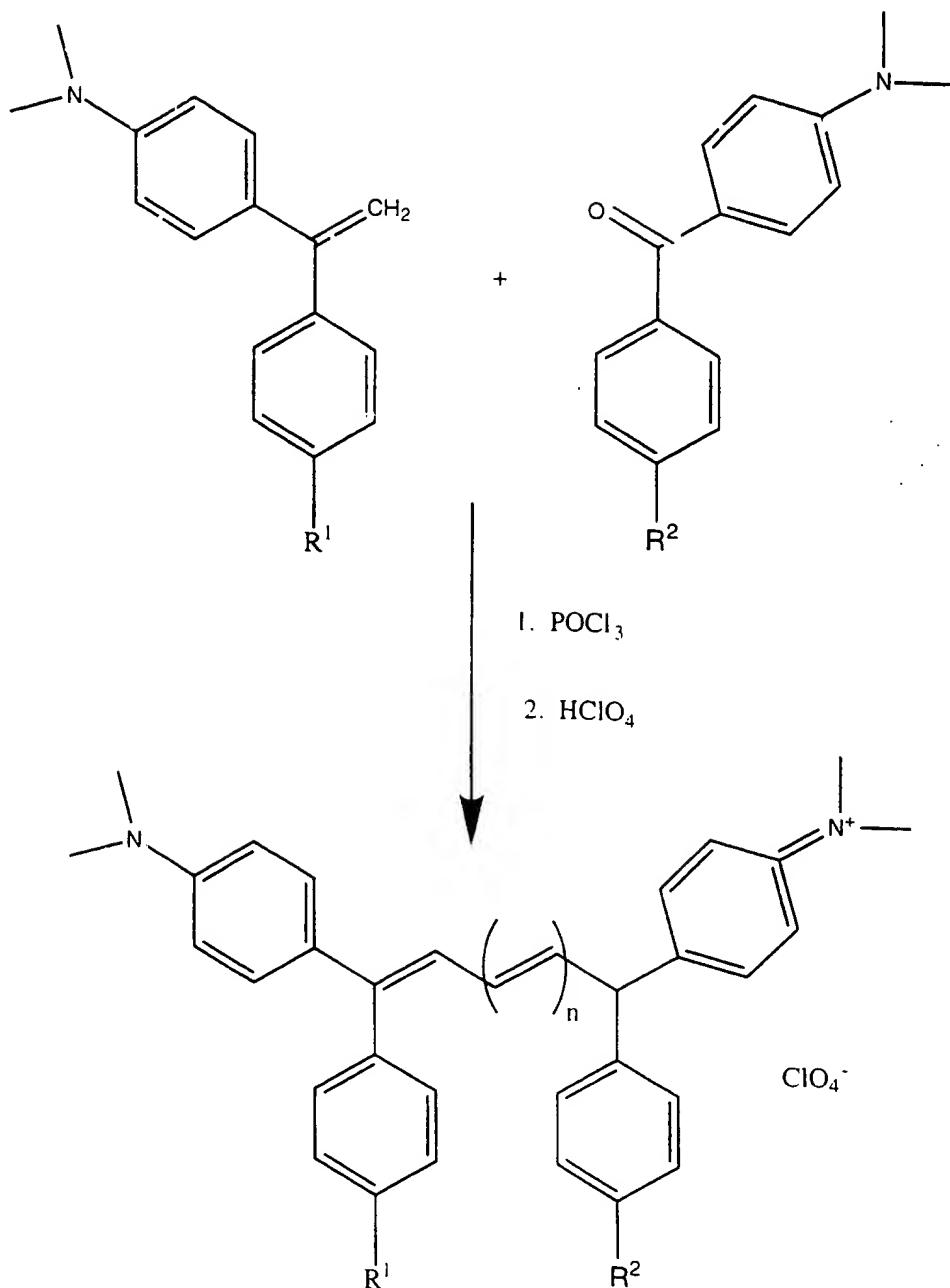
$n = 0, R^1 = R^2 = O\text{-PEG}\text{-OCH}_3$

$n = 0, R^1 = O\text{-PEG}\text{-OCH}_3; R^2 = H$

$n = 0, R^1 = O\text{-PEG}\text{-OCH}_3; R^2 = \text{Cl}, \text{Br}, \text{I}, \text{F}$

$n = 0, R^1 = O\text{-PEG}\text{-OCH}_3; R^2 = \text{linker group}$

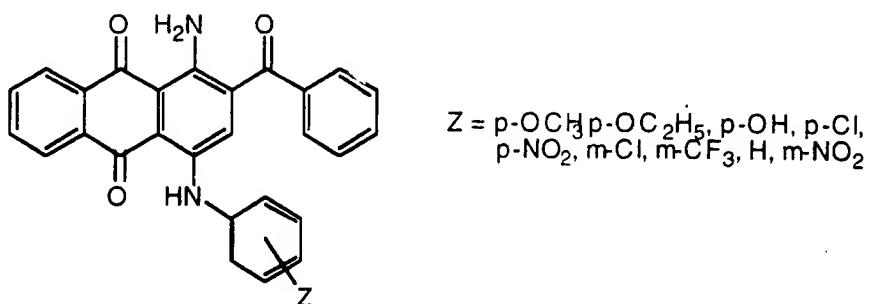
For PEG Pentamethines and PEG Heptamethines:



5 wherein n is 1 or 2 and R is and O-PEG-OCH<sub>3</sub>.

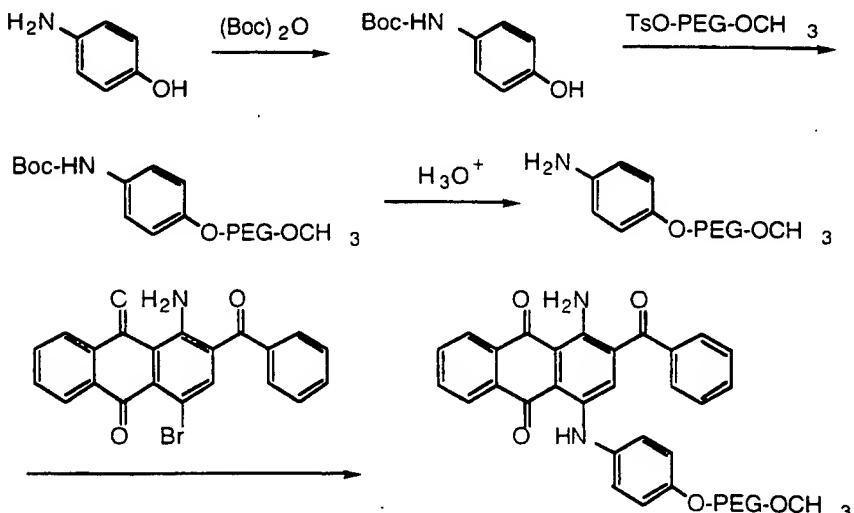
Example 2: Quinoid Dyes:

Quinoid dyes can be coupled to one or more polyoxyhydrocarbyl moieties and include 1-Amino-2-benzoyl-4-(aryl amino)anthraquinones such as those described by L. Boguslavskaya, V. I. Gudzenko Z. Organ. Khim. 1968, 4, 101-104, incorporated herein by reference in its entirety, including any drawings and preferably have the structure:



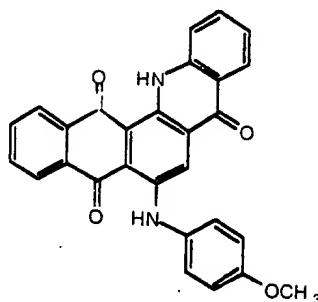
These compounds have the following spectral properties:  $\lambda_{\max}$  ranges from ca. 670 to 720 nm and  $\epsilon$  ranges from ca. 100,000 to 200,000.

PEG containing conjugates of such compounds can be synthesized as follows:

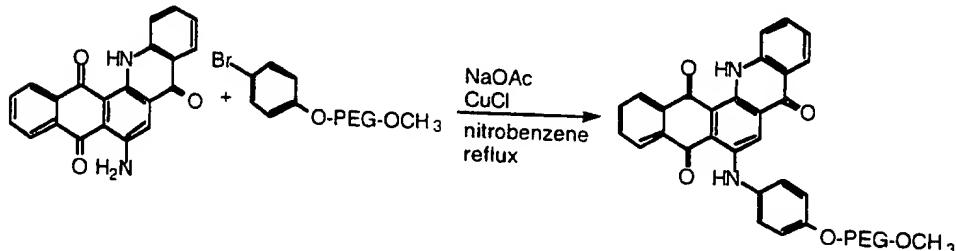


30

Other quinoid dyes that can be used in the present invention include 6-(Arylamino)naphth [2,3,c]acridan-5,8,14-triones such as those described by L. Boguslavskaya, V. I. Gudzenko Z. Organ. Khim. 1968, 4, 101-104 and preferably have the structure:



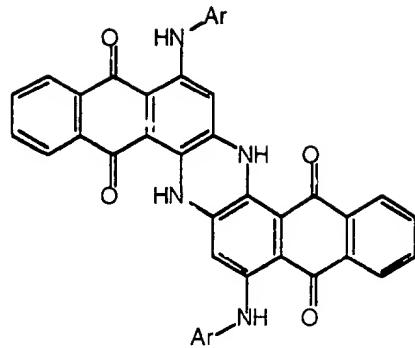
Such compounds have the following spectral properties:  $\lambda_{\text{max}} = 732$  nm. PEG containing conjugates may be synthesized as follows:



Example 3: Indanthrene Dyes:

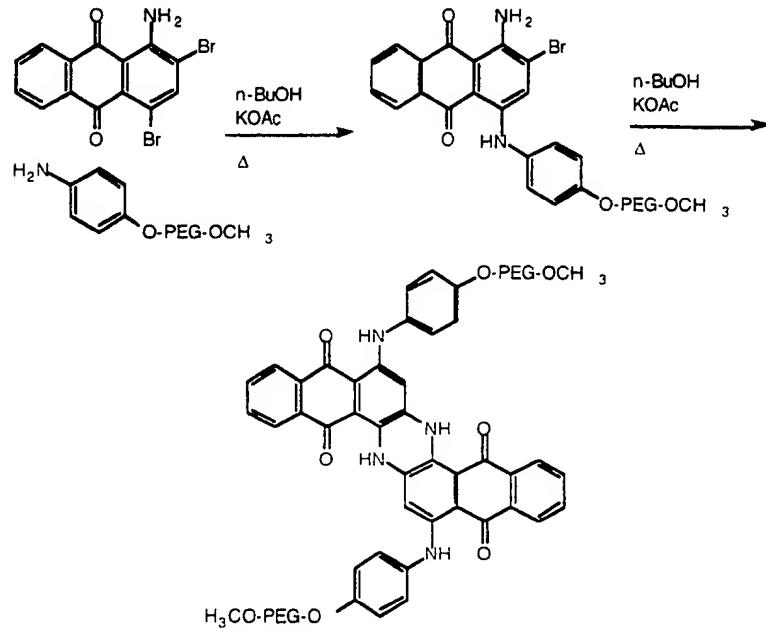
Indanthrene dyes may also be coupled to polyoxyhydrocarbyl moieties and include those described in A. Tundo Ann. Chim. (Rome) 1957, 47, 291-298, incorporated herein by reference in its entirety. Such compounds preferably have the general structure:

31



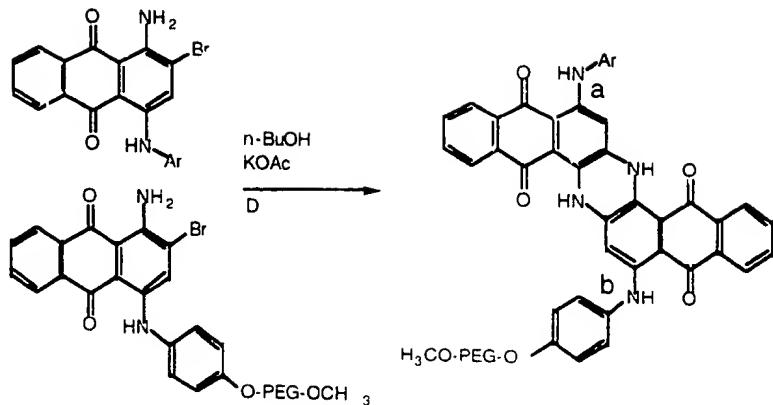
Such compounds have the following spectral properties:  $\lambda_{\text{max}}$  ranges from ca. 715 nm to 790 nm. log  $\epsilon$  ranges from ca. 4.0 to 4.6.

PEG containing conjugates may be synthesized as follows:



10

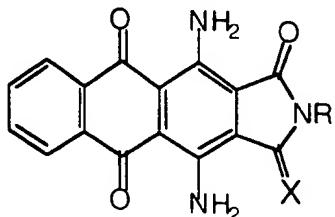
Mixed condensations which incorporate a single PEG residue at position "a" and a suitably functionalized aryl residue at position "b" are also possible using this route.



Example 4: 1,4-Diaminoanthraquinone-2,3-dicarboxamides:

5 1,4-Diaminoanthraquinone-2,3-dicarboxamides may also be used and include those described in: *Chemical Abstracts* 1984, 100, 193530n, incorporated herein by reference in its entirety, including any drawings. Such compounds preferably have the structure:

10

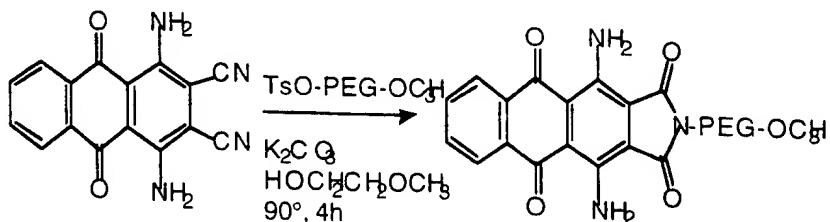


X = O, S, NH, NR'

15

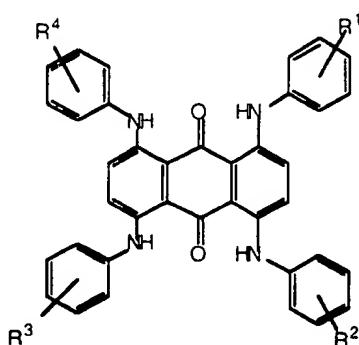
Such compounds have the following spectral properties: for R = n-C<sub>6</sub>H<sub>13</sub>, λ<sub>max</sub> = 763 in the liquid crystalline phase.

PEG conjugates may be synthesized as follows:



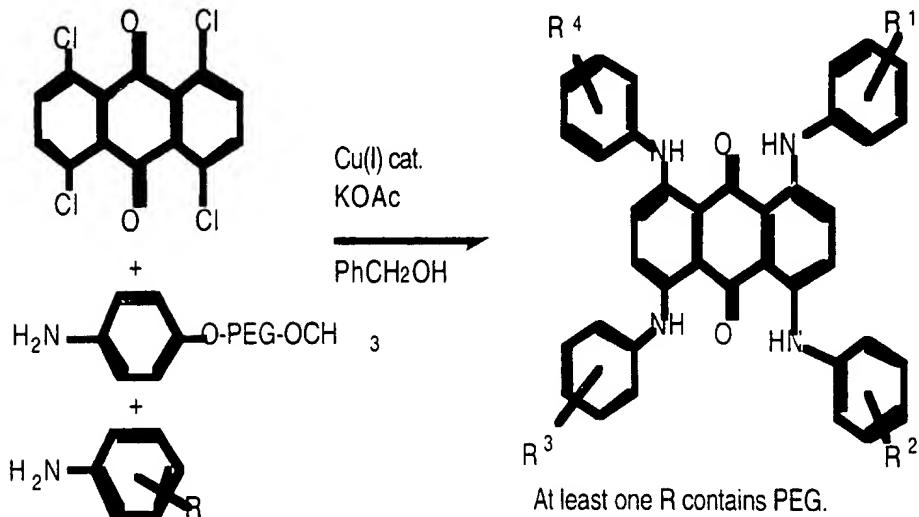
Example 5: Tetraaminoanthraquinones:

Tetraaminoanthraquinones may also be used and include those described in T. Ohyamata, K. Takuma; S. Kuroda, H. Aiga. Eur. Pat. Appl. EP 23,184, 05 Jul. 1989, incorporated herein by reference in its entirety. Such compounds preferably have the structure:

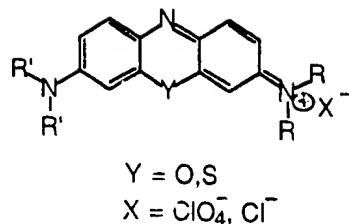


Such compounds have the following spectral properties: for R = alkyl,  $\lambda_{\text{max}} = 741$  in EtOH.

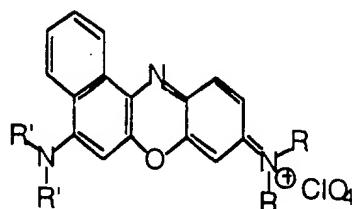
PEG conjugates may be synthesized as follows:

Example 6: Azine Dyes

Azine dyes may also be used and preferably have the structure:



More preferably the dye is Nile Blue A perchlorate and has the structure:



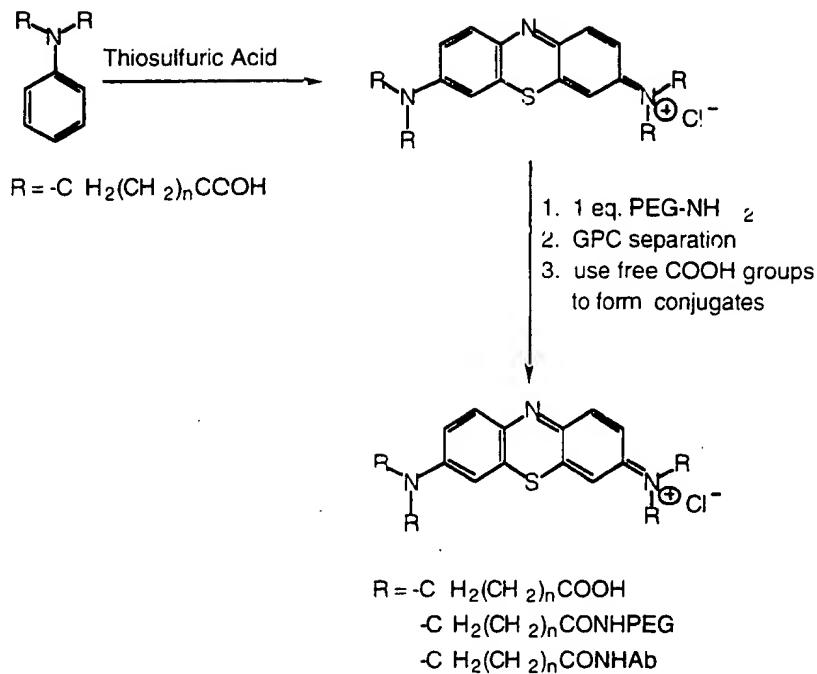
Such compounds have the following spectral properties:

R	R'	Name	Abs. $\lambda_{\max}$ (nm)	Fluor. $\lambda_{\max}$ (nm)	$\epsilon \times 10^4$ (L/mol/cm)
Ethyl	Ethyl	Oxazine 1	643	658	12.30
Ethyl	H	Nile Blue A	627	660	7.68
Methyl	Methyl	Methylene Blue	695*	885*	NA
H	H	Thionin	695*	850*	NA
Methyl	H	Toluidine Blue	695*	848*	NA

10 \* From: "Laser Dyes", Mitsuo Maeda. For the starred entries, Abs.  $\lambda_{\max}$  is that of the ruby pumping laser, and Fluoro, lambda max is the lasing wavelength.

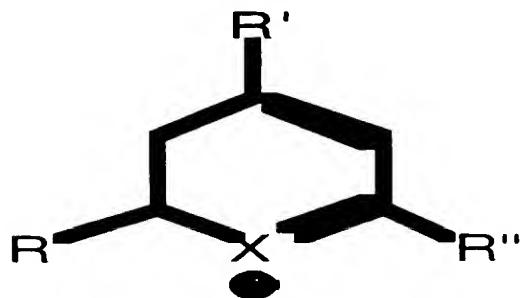
PEG conjugates may be synthesized as follows and by using techniques described in Processes of Dye Chemistry (Interscience, New York) 1949.

35



Example 7: Pyrylium and Thiopyrylium Dyes

Pyrylium and Thiopyrylium Dyes may also be used and  
 5 preferably have the structure:



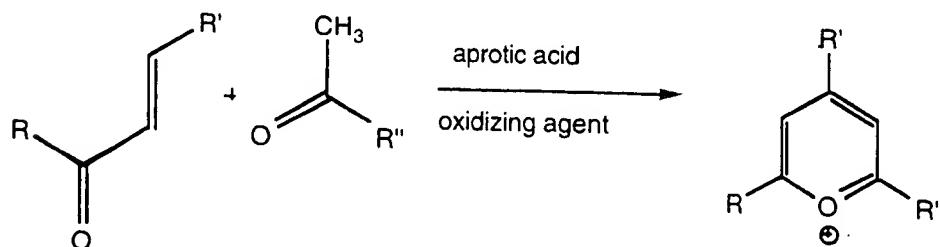
$\text{X} = \text{O}, \text{S}$   
 $\text{R}, \text{R}', \text{R}'' = \text{alkyl, aryl}$   
 or enol ether

Such compounds may be synthesized as follows and  
 10 using techniques described in A. T. Balaban, W. Schroth,

G. W. Fischer, *Adv. Heterocyclic Chem.* 1969, 10, 241; and A. T. Balaban, A. Dinculescu, G. N. Dorofeenko, G. W. Fischer, A. V. Koblik, V. V. Meqheritskii, W. Schroth "Pyrylium Salts. Syntheses, Reactions and Physical Properties", *Adv. Heterocyclic Chem., Suppl. 2*, Ed. A. R. Katritzky, Academic Press, New York, 1982, both of which are incorporated herein by reference in their entirety, including any drawings.

5

10



Thiopyrylium analogs are made by treating the pyrylium salts with sodium sulfide.

Such compounds have the following properties:

15

R	R'	R''	$\lambda$ (nm)	$\epsilon \times 10^4$
Ph	Ph	-CH=CH-(4-OCH <sub>3</sub> -Ph)	530 <sup>a</sup>	2.7
Ph	Ph	- (CH=CH) <sub>2</sub> -(4-OCH <sub>3</sub> -Ph)	580 <sup>a</sup>	4.7
Ph	Ph	- (CH=CH) <sub>3</sub> -(4-OCH <sub>3</sub> -Ph)	630 <sup>a</sup>	4.4
Ph	Ph	- (CH=CH) <sub>4</sub> -(4-OCH <sub>3</sub> -Ph)	670 <sup>a</sup>	7.7
Ph	Ph	- (CH=CH) <sub>5</sub> -(4-OCH <sub>3</sub> -Ph)	700 <sup>a</sup>	6.0
Ph	Ph	-CH=CH-OEt	725 <sup>b</sup>	4.0
p-OMe-Ph	p-OMe-Ph	-CH=CH-OH	724 <sup>b</sup>	3.5
-CH <sub>2</sub> Ph	-CH <sub>2</sub> Ph	-CH=CH-OEt	726 <sup>b</sup>	3.4

<sup>a</sup> Spectrum measured in dichloromethane

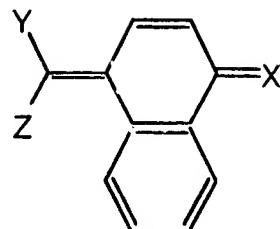
<sup>b</sup> Spectrum measured in acetic acid

20

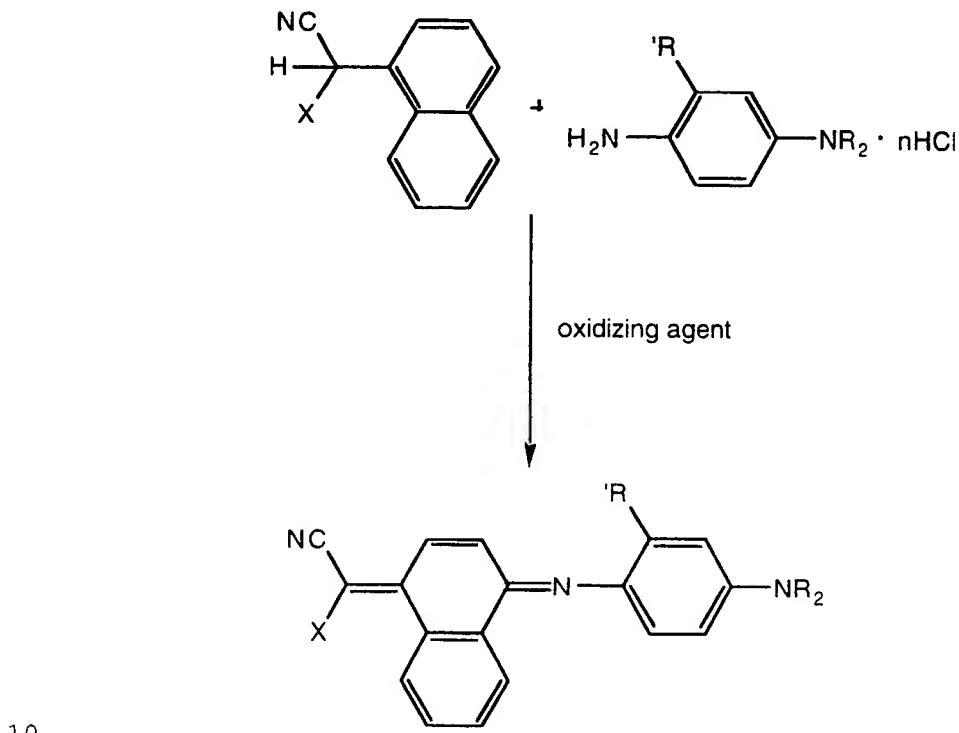
#### Example 8: Naphthoquinone Methides

Naphthoquinone Methides may also be used and preferably have the structure:

37



Such compounds may be synthesized as follows and using the techniques described in: The Chemistry of the Quinoid Compounds, ed., S. Patai, Y. Kubo, F. Mori, K. Yoshida *Chemistry Letters*, 1987, 1761 - 1762, incorporated herein by reference in its entirety, including any drawings.



10

Such compounds have the following properties:

15

38

X	Y	Z	$\lambda$ (nm)	$\epsilon \times 10^4$
-N-(2-Me-4-N,N'-Diethylamino-C <sub>6</sub> H <sub>3</sub> )	CN	CN	761 <sup>a</sup>	3.1
-N-(4-N,N'-Dimethylamino-C <sub>6</sub> H <sub>4</sub> )	CN	CN	722 <sup>a</sup>	2.5
-N-(2-Me-4-N,N'-Diethylamino-C <sub>6</sub> H <sub>3</sub> )	CN	CONH <sub>2</sub>	754 <sup>a</sup>	2.9

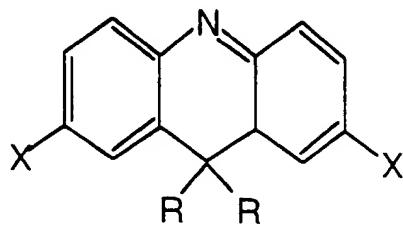
<sup>a</sup> Spectra measured in chloroform.

PEG conjugates could be synthesized following the above route with R or 'R = PEG.

5

Example 9: Carbazines (also called acridans)

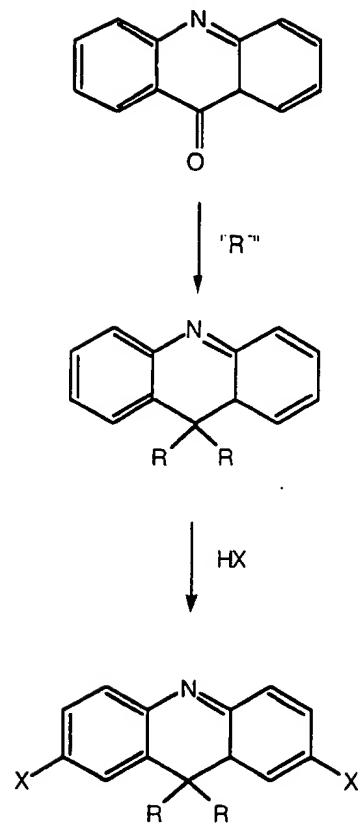
Carbazines may also be used and include those described in *Heterocyclic Compounds* vol. 9 "Acridines," Ed. R. M. Acheson, John Wiley and Sons, Inc., New York, 1973, incorporated herein by reference in its entirety, including any drawings. Such compounds preferably have the structure:



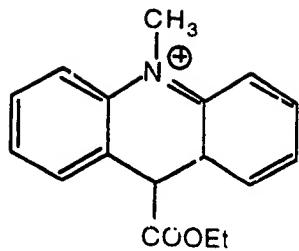
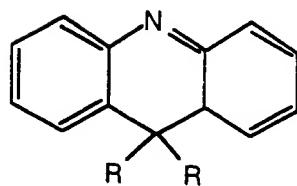
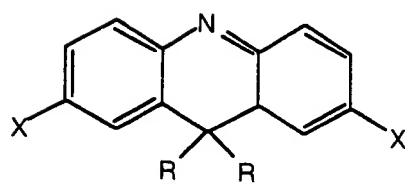
15

Such compounds may be synthesized as follows:

39



40

 $\downarrow \text{RX}$  $\downarrow \text{HX}$ 

In these reactions one or more of the R groups may be  
5 PEG.

Example 10: Non-Specific Binding Tests

Cyanine dyes have a long history as sensitizers in  
10 the photographic process. Recently, water soluble cyanine  
dyes with chemically reactive groups have become  
commercially available as fluorescent labels. One of  
these dyes, Cy 5.5 has an absorption maximum at about 680  
nm and emits just over 700nm.

A sample of Cy 5.5 at 1.7 micromolar concentration  
15 was kept in digoxin probe diluent for 72 hr. after which

time the original active ester form should be converted to the free carboxyl form. Tests for non-specific binding were made with our standard serum panel and showed pronounced effects of serum or serum components on both 5 the intensity and polarization. These effects are manifest as non-linearity in the intensity as a function of concentration (Tables I and II) and in a variation of intensity with the type and concentration of serum component (Table III) with the polarization being elevated 10 by non-specific binding.

Table I - Cy 5.5 in Digoxin Diluent

	Concentration nM	Fluorescence Intensity	Milli-polarization
			Units
15	170	416279	82.8
	17	580613	outside readability
	1.7	150069	129.0
	0.17	38842	122.6

Table II - Cy 5.5 in TDx Buffer

20	170	1074511	-34.2
	17	1338283	outside readability
	1.7	150069	129.0
	0.17	22280	130.2

Table III - Cy 5.5 at 1.7nM + Additives

	Additive	Intensity	Millipolarization
			units
30	0.5%BSA	665960	285.5
	5% Bov. Ser.	555277	260.1
	0.25% BGG	326543	191.2
	5% PHS	608260	282.9
	5% PHS	383004	231.9

35 The bis-active ester can be coupled to diisopropyl amine and the binding of this form to serum examined. This test should be indicative of the behavior to be

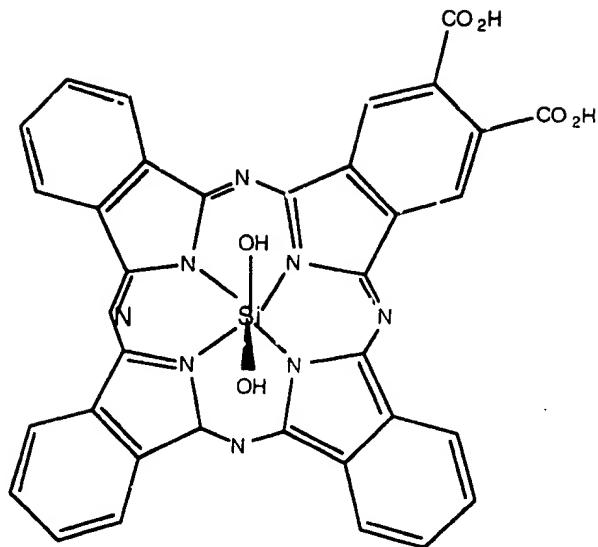
expected from bio-probes made from Cy 5.5. A digoxin probe can be made by reacting the bis-functional dye with an excess of 3-amino-3-deoxydigoxigenin (DIG) so that each dye molecule will be combined with two digoxigenin molecules. This probe can then be tested against the serum panel and against anti-digoxin to determine its properties as digoxin probe.

In order to suppress non-specific binding Cy 5.5 can be coupled to polyethylene glycol monomethyl ether (PEG) as we normally do for phthalocyanines such as those used in making La Jolla Blue. If this bis-reactive form is used then the result will be to couple two PEG molecules to each dye molecule. If the mono-reactive form of the dye is used only one PEG can be coupled so that information on the amount of PEG necessary to achieve the desired degree of suppression will be obtained.

A hybrid molecule containing one digoxigenin molecule and one PEG can be made by first reacting the bis-reactive dye with a limited amount of DIG to obtain a certain of monosubstitution followed by reaction with excess PEG and purification by high performance liquid chromatography (HPLC).

Example 11: Relationship Between Non-Specific Binding and Solubility

Nonspecific binding and water solubility do not necessarily go hand in hand, but instead dye molecules can be monomeric in aqueous solution and yet show powerful nonspecific binding. As an example from our work, the binding constant for a bare phthalocyanine of structure shown as Formula A below reacting with human serum albumin (HSA) was calculated from measurements of transient state fluorescence in potassium phosphate buffer at pH 8.0 with various concentrations of added HSA.



Formula A (Bare dicarboxy silicon phthalocyanine)

Similar measurements on the dye of Formula A

5 chemically modified by attaching a polyethylene glycol molecule to each of the silanol OH groups to make the dye La Jolla Blue were made in the same buffer and instrument. The results are shown in Table IV below.

10

## Table IV:

Transient State Fluorescence Intensity and Polarization Measurements on Formula A Dye and on La Jolla Blue with or without Added HSA.

15

Dye concentration in both cases was 3.1 E-10 M.

## Formula A Dye

	% HSA	Intensity	Polarization (mP)
	0	164696	2.4
20	0.002	105080	63.5
	0.02	67714	153.9
	0.2	63672	192.8
	2.0	78321	191.7
	4.0	81328	201.1

## La Jolla Blue

	0	85743	1.3
	0.002	84887	4.8
5	0.02	82718	18.1
	0.2	81073	20.8
	2.0	56085	35.8
	4.0	71593	71.8

10           The data of Table IV can be used to determine the HSA concentration at which the dye is half free and half bound. For the bare dye and for La Jolla Blue this concentration is 3.2 E-7 M and 5.0 E-4 M, respectively, making the binding constants 3E6 and 2E3 M<sup>-1</sup>, respectively.  
15           In other words, the introduction of PEG molecules, in this case of formula weight 2000, is capable of decreasing the nonspecific binding constant by a factor of over a thousand.

Claims:

1. A detectably labeled marker component which comprises:

5 (a) a fluorophore moiety comprising a luminescent substantially planar molecular structure having an excitation wavelength of at least about 500 nanometers; coupled to

10 (b) one or more polyoxyhydrocarbyl moieties, wherein said marker component has decreased non-specific binding to components of serum as compared to the bare fluorophore moiety when it is not coupled to one or more polyoxyhydrocarbyl moieties.

15 2. A marker component according to claim 1 wherein said fluorophore moiety has an excitation wavelength of about 600 to 800 nanometers.

20 3. A marker component according to claim 2 wherein said fluorophore moiety has an excitation wavelength of at least 650 nanometers.

25 4. A marker component according to claim 1 wherein said marker component has substantially similar intensity, decay time and relative magnitudes of the polarized components in the presence and absence of serum.

30 5. A marker component according to claim 1 wherein said fluorophore moiety alone has a binding constant that is at least 50 times greater than the binding constant of the complete marker component.

35 6. A marker component according to claim 5 wherein said fluorophore moiety alone has a binding constant that is at least 100 times greater than the binding constant of the complete marker component.

7. A marker component according to claim 6 wherein said fluorophore moiety alone has a binding constant that is at least 500 times greater than the binding constant of the complete marker component.

5

8. A marker component according to claim 7 wherein said fluorophore moiety alone has a binding constant that is at least 1,000 times greater than the binding constant of the complete marker component.

10

9. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of: (1) aryl terminated polymethine dyes; (2) quinoid dyes; (3) indanthrene dyes; (4) 1,4-diaminoanthraquinone-2,3-dicarboxamides; (5) tetraaminoanthraquinones; (6) azine dyes; (7) pyrylium or thiopyrylium dyes; and (8) naphthoquinone methides.

20

10. A marker component according to claim 1 wherein said polyoxyhydrocarbyl moieties are selected from polyethers, polyols, water soluble carbohydrates, water soluble carbohydrate derivatives, and water soluble polymers.

25

11. A marker component according to claim 10 wherein said polyoxyhydrocarbyl moieties comprise polyethylene glycol or polyethylene glycol derivatives.

30

12. A marker component according to claim 11 wherein each said polyoxyhydrocarbyl moieties has a molecular weight of about 200 to about 20,000.

35

13. A marker component according to claim 12 wherein each of said polyoxyhydrocarbyl moieties has a molecular weight between 2,000 and 20,000.

14. A marker component according to claim 13 wherein each of said polyoxyhydrocarbyl moieties has a molecular weight between 4,000 and 15,000.

5       15. A market ccomponent according to claim 14 wherein each of said polyoxyhydrocarbyl moieties has a molecular weight between 8,000 and 10,000.

10      16. A marker component according to claim 1 which in the presence of serum components in aqueous solution is characterized by transient state fluorescence emission having parallel and perpendicular components of substantially the same intensities as without serum.

15      17. A marker component according to claim 9 wherein said fluorophore moiety is an aryl terminated polymethine dye.

20      18. A marker component according to claim 17 wherein said aryl terminated polymethine dye is a trimethine.

25      19. A marker component according to claim 17 wherein said aryl terminated polymethine dye is a pentamethine.

30      20. A marker component according to claim 17 wherein said aryl terminated polymethine dye is a heptamethine.

35      21. A marker component according to claim 9 wherein said fluorophore moiety is an quinoid dye.

        22. A marker component according to claim 21 wherein said quinoid dye is a 1-Amino-2-benzoyl-4-(arylamino)anthraquinone.

        23. A marker component according to claim 9 wherein said quinoid dye is a 6-(Arylamino)napth [2,3,c]acridan-5,8,14-trione.

24. A marker component according to claim 9 wherein said fluorophore moiety is an indanthrene dye.

5       25. A marker component according to claim 9 wherein said fluorophore moiety is a 1,4-diaminoanthraquinone-2,3-dicarboxamide.

10      26. A marker component according to claim 9 wherein said fluorophore moiety is a tetraaminoanthraquinone.

27. A marker component according to claim 9 wherein said fluorophore moiety is an azine dye.

15      28. A marker component according to claim 9 wherein said fluorophore moiety is a pyrylium or thiopyrylium dye.

20      29. A marker component according to claim 9 wherein said fluorophore moiety is a naphthoquinone methide.

30. A marker component according to claim 9 wherein said fluorophore moiety is a carbazine.

25      31. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 32221; (2) 32222; (3) 32223; (4) 32224; and (5) 32225.

30      32. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 35512; (2) 35513; (3) 35514; (4) 35517; (5) 35518; and (6) 33519.

35      33. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 35523; (2) 35524; (3) 35525; (4) 35526; (5) 35527; (6) 35528; (7) 35529; and (8) 35530.

34. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 35533; (2) 35535; (3) 35536; and (4) 35538.

5

35. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 50024; (2) 50025; and (3) 50032.

10

36. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 62303; (2) 62401; (3) 62403a; (4) 62403b; (5) 62402; and (6) 62411.

15

37. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 64102; (2) 64104; (3) 64106; (4) 64303a; (5) 64304; (6) 64311; (7) 64311; (8) 64312; and (9) 65212.

20

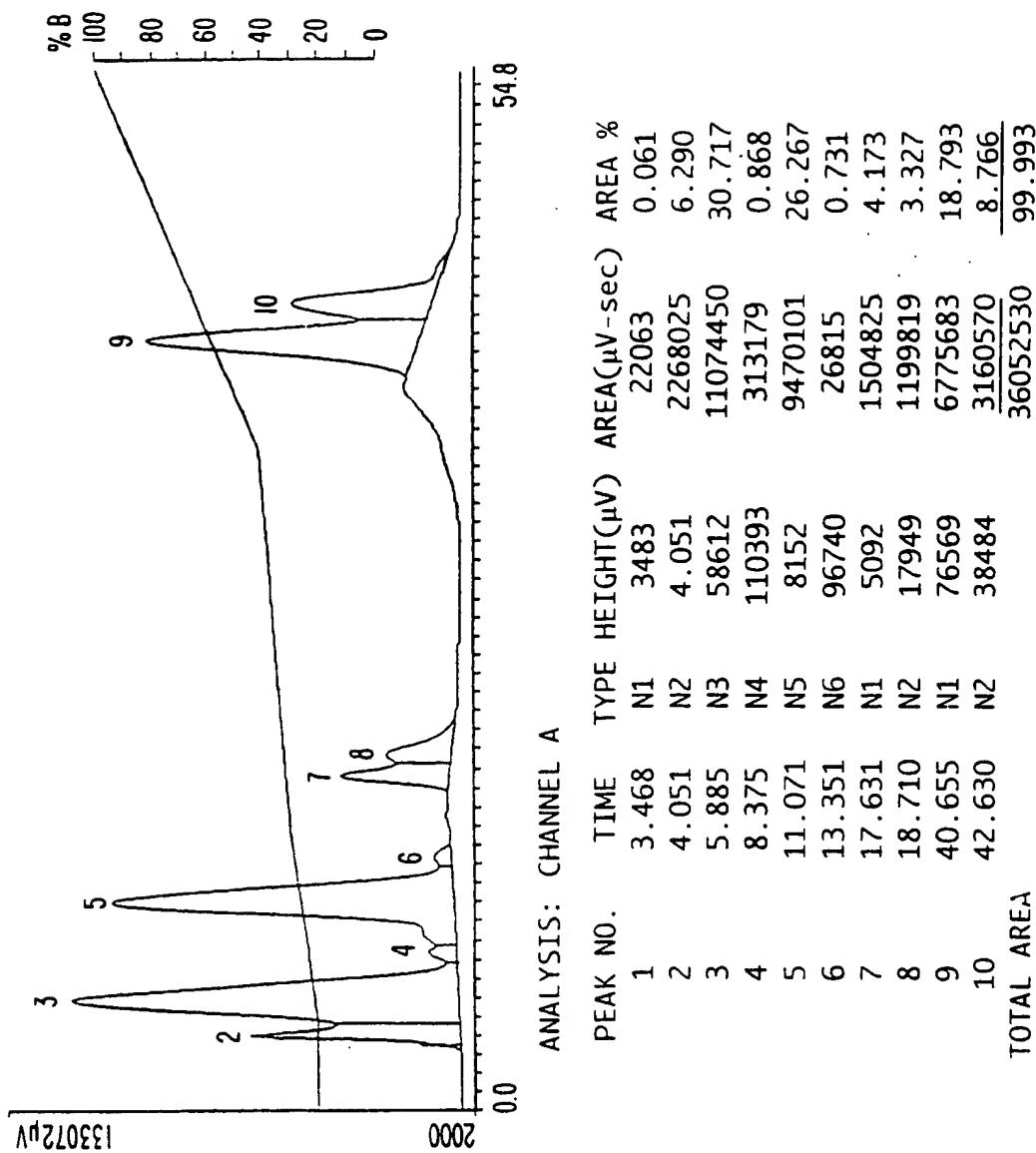
38. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 66001; (2) 66002; (3) 66004; (4) 66005; (5) 66008; (6) 66009; (7) 66013; (8) 66014; (9) 66015; and (10) 66204.

25

39. A marker component according to claim 1 wherein said fluorophore is selected from the group consisting of compounds: (1) 66206; (2) 66210; (3) 67102; (4) 67422; and (5) 91022.

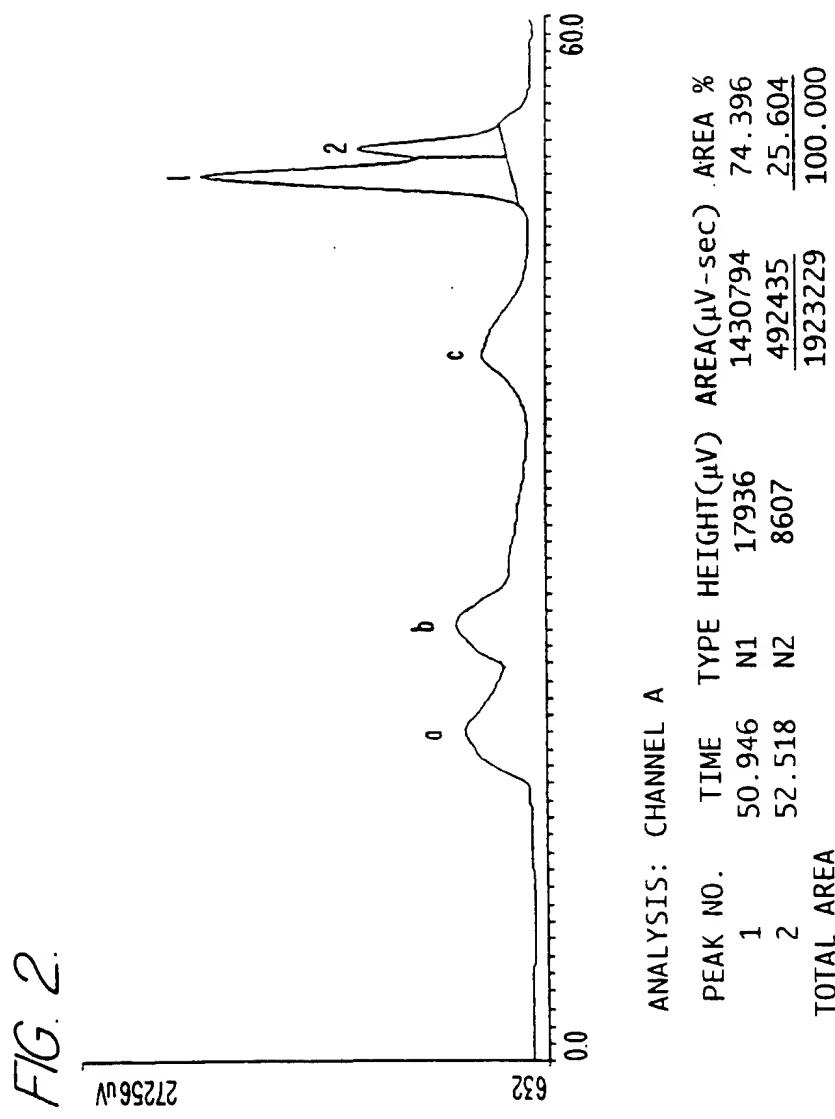
30

FIG. 1.



SUBSTITUTE SHEET (RULE 26)

2/28



SUBSTITUTE SHEET (RULE 26)

3/28

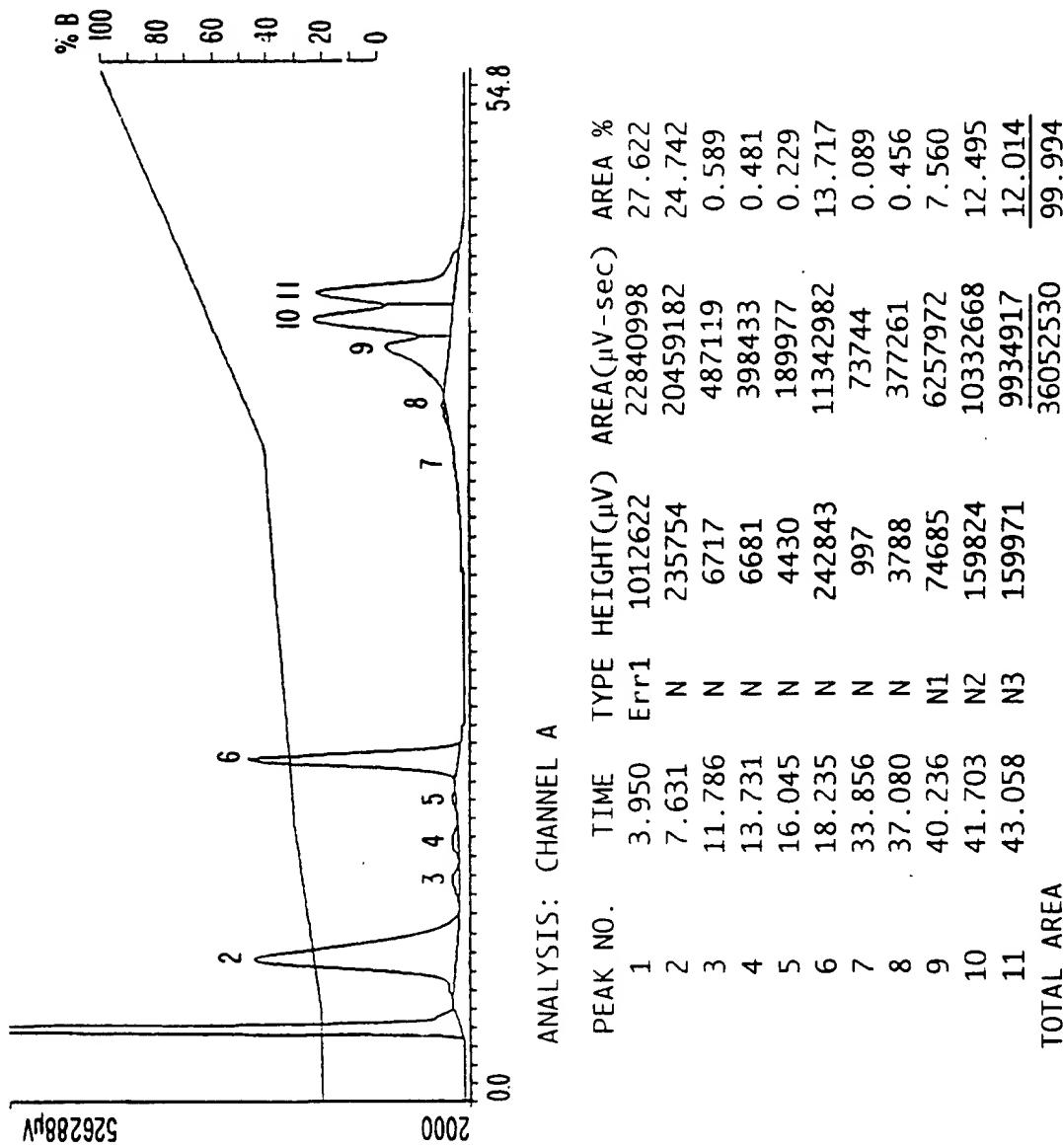
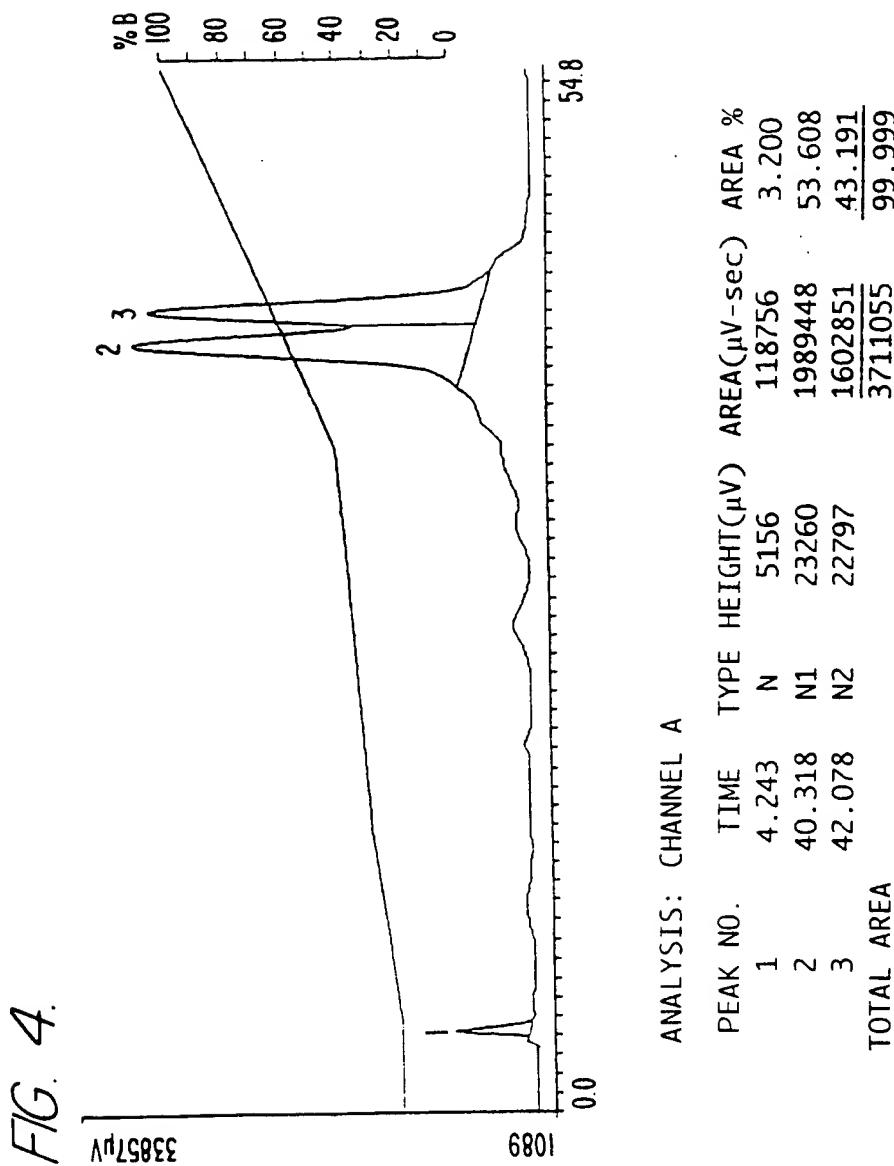


FIG. 3.

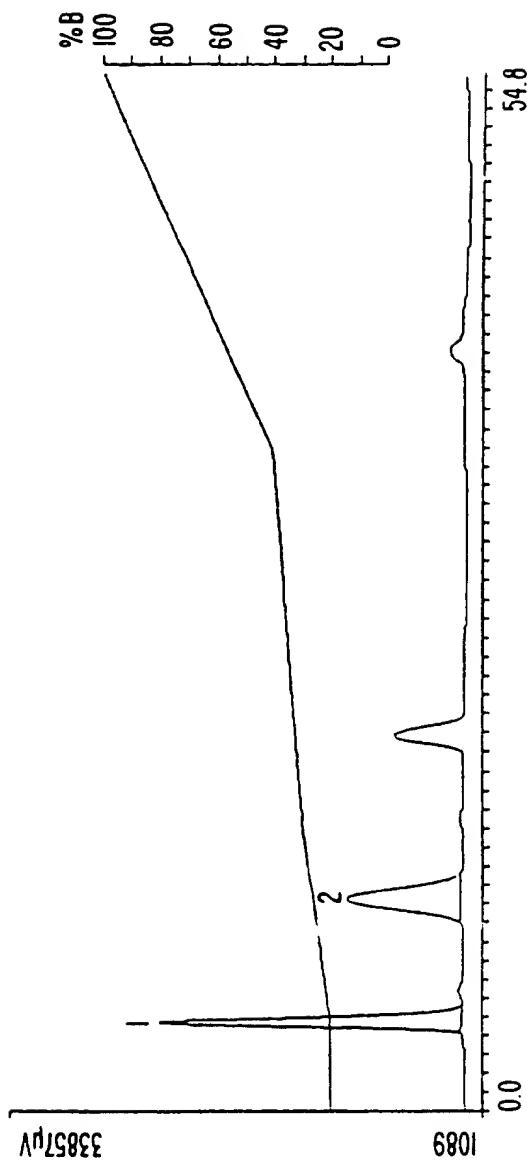
4/28



SUBSTITUTE SHEET (RULE 26)

5/28

FIG. 5.

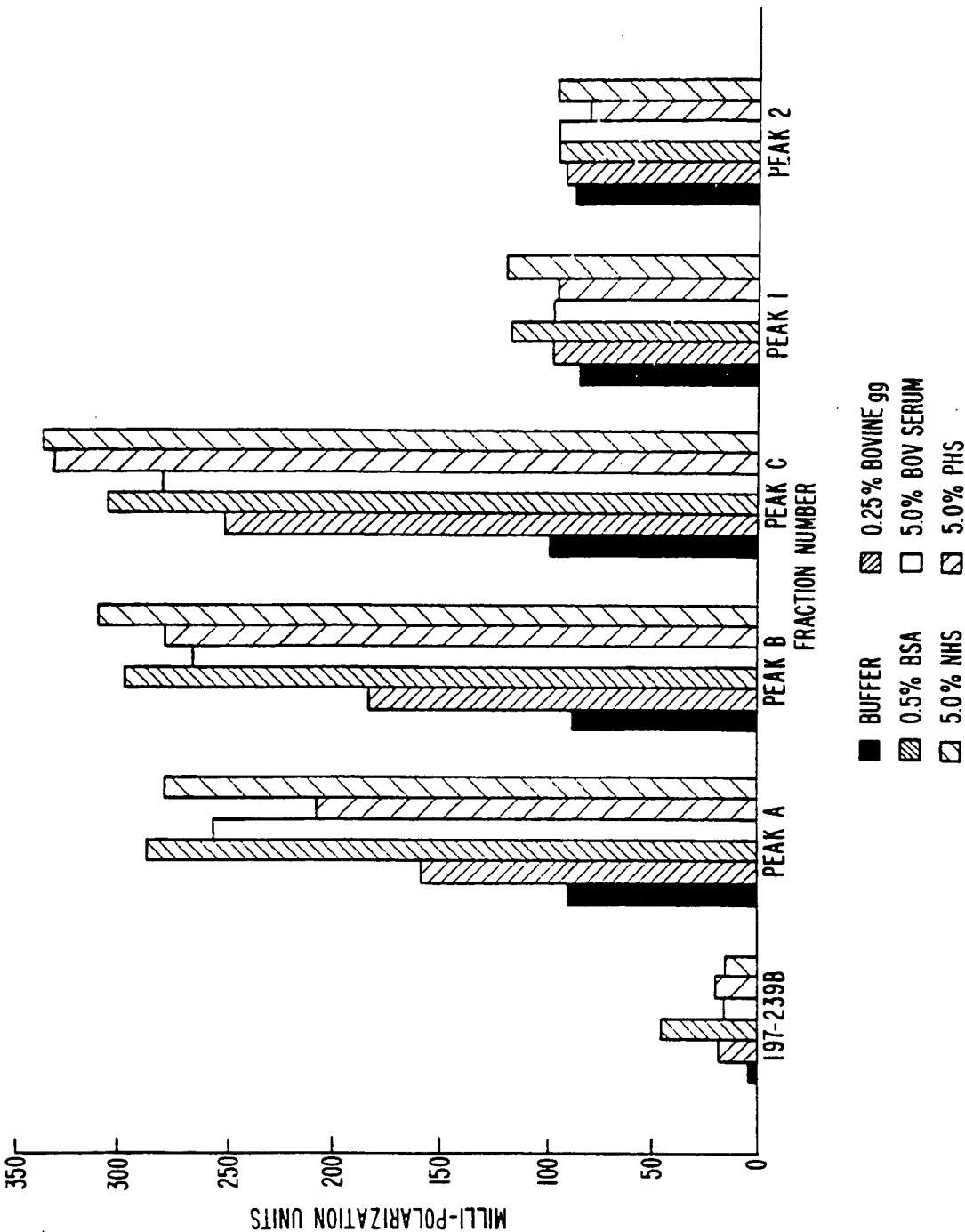


## ANALYSIS: CHANNEL A

PEAK NO.	TIME	TYPE	HEIGHT(μV)	AREA(μV-sec)	AREA %
1	4.720	N	21095	700640	53.339
2	11.290	N	8187	<u>612906</u>	<u>46.660</u>
TOTAL AREA				1313546	99.999

SUBSTITUTE SHEET (RULE 26)

6/28



SUBSTITUTE SHEET (RULE 26)

7/28

*FIG. 7a.*

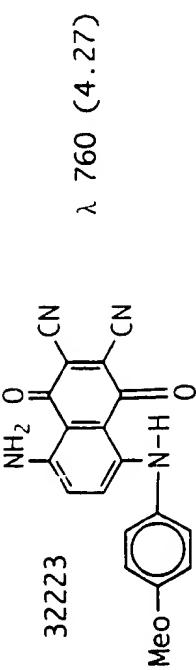
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
4-(4H-THIOPYRAN-4-YLIDENE-4-H-THIOPYRAN)		$\lambda$ 726 (4.17)
QUINONE METHIDE, TRISPHENOQUINONE (A TRIQUINOCYCLOPROPANE)		$\lambda$ 760 (4.71)
8-PHENYLAMINO-5-AMINO-2,3-DICYANO- 1,4-NAPHTHOQUINONE		$\lambda$ 758 (4.24) 735 (3.95), 786 (3.99).
8-(4'METHYLPHENYLAMINO)-5-AMINO-2,3- DICYANO-1,4-NAPHTHOQUINONE		$\lambda$ 760 (4.27)

*FIG. 7b.*

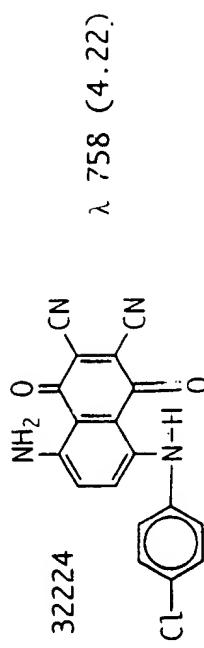
COMPOUND NAME

WAVELENGTH

8-(4' METHOXYPHENYLAMINO)-  
5-AMINO-2,3-DICYANO-1,4-  
NAPHTHOQUINONE

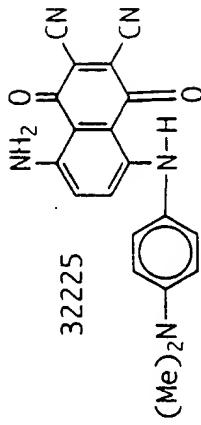


8-(4' CHLOROPHENYLAMINO)-5-AMINO-  
2,3-DICYANO-1,4-NAPHTHOQUINONE



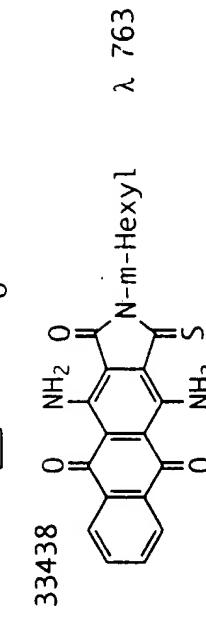
$\lambda$  760 (4.27)

8-(4' -DIMETHYLAMINOPHENYLAMINO-5-AMINO-  
2,3-DICYANO-1,4-NAPHTHOQUINONE



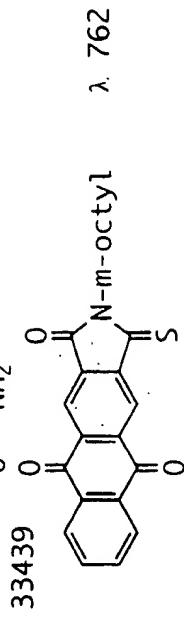
$\lambda$  758 (4.22)

1,4-DIAMINOANTHRAQUINONE-(N-m-hexyl)-  
3'-THIOXO-2,3-DICARBOXIMIDE

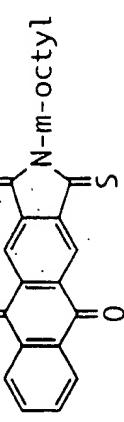


$\lambda$  802 (4.28)

1,4-DIAMINOANTHRAQUINONE-(N-m-octyl)-  
3'-THIOXO-2,3-DICARBOXIMIDE



$\lambda$  763



$\lambda$  762

9/28

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
ANTHRAQUINONE-1,4-DISULFENIC ACID	<p>34102</p>	$\lambda$ 425 (3.82), 740 (3.66) (BASIC SOLN.)
ANTHRAQUINONE-1,5-DISULFENIA ACID	<p>34103</p>	$\lambda$ 419 (3.92), 732 (3.79) (BASIC SOLN.)
ANTHRAQUINONE-1,4-DISELENIC ACID	<p>34105</p>	$\lambda$ 439 (3.88), 742 (3.79)
6,15-BIS(ANILIO)INDAURHRENE	<p>35509</p>	$\lambda$ 445-460 (4.12), 715 (4.48), 790 (4.74)

FIG. 7C.

SUBSTITUTE SHEET (RULE 26)

10/28

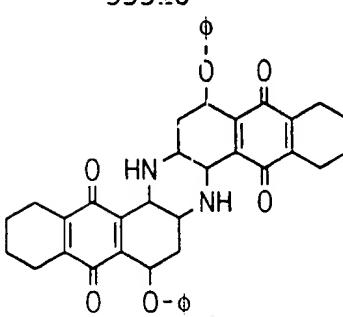
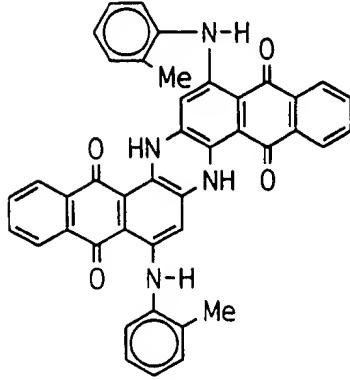
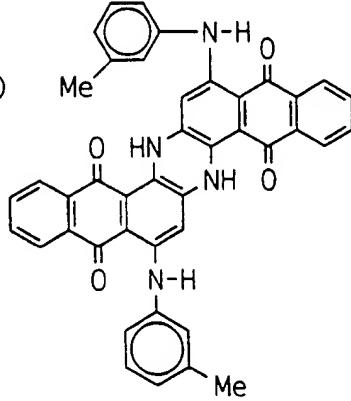
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
8,17-DIPHENOXYINDAURHRENE	35510 	
8,17-BIS(2-TOLUIDINO) INDANTHRENE	35512 	$\lambda$ 715 (4.51), 785 (4.66)
8,17-BIS(3-TOLUIDINO) INDANTHRENE	35513 	$\lambda$ 455-466 (4.15) 715 (4.48), 790 (4.75)

FIG. 7d.

SUBSTITUTE SHEET (RULE 26)

11/28

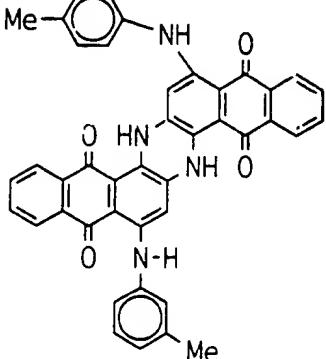
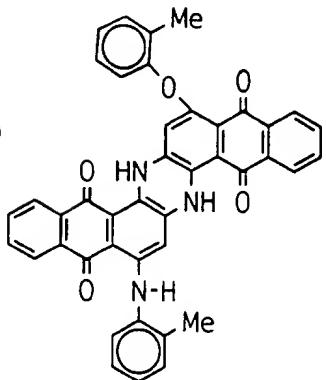
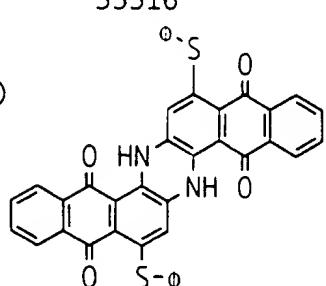
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
8,17-DIPHENOXY-INDAURHRENE	35514 	$\lambda$ 440-455 (3.94) 715 (4.27), 790 (4.49)
8,17-BIS(2-TOLYLOXY)INDANTHRENE	35515 	$\lambda$ 610 (4.03), 680 (4.39), 735 (4.47)
8,17-BIS(PHENYLTHIO)INDANTHRENE	35516 	$\lambda$ 615 (4.01), 685 (4.40), 750 (4.60)

FIG. 7e.

SUBSTITUTE SHEET (RULE 26)

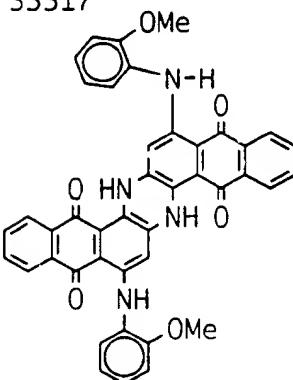
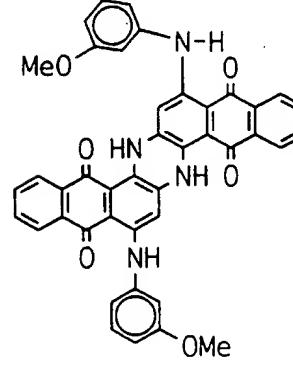
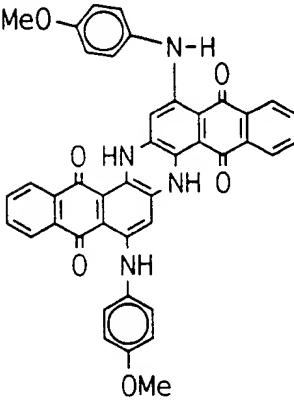
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
8,17-BIS(2-ANISIDINO) INDANTHRENE	35517 	$\lambda$ 465 (3.99), 715-720 (4.45), 790 (4.63)
8,17-BIS(3-ANISIDINO) INDANTHRENE	35518 	$\lambda$ 465-470 (3.98), 720-725
8,17-BIS(4-ANISIDINO) INDANTHRENE	35519 	$\lambda$ 465 (3.99), 715-720 (4.47), 790 (4.69)

FIG. 7f

13/28

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
8,17-BIS(2-METHOXY-PHENOXY) INDANTHRENE	35520 	$\lambda$ 610 (4.05), 675-680 (4.36), 735-740 (4.44).
8,17-BIS(4-METHOXY-PHENOXY) INDANTHRENE	35521 	$\lambda$ 610 (4.00), 675-680 (4.43), 735-740 (4.43).
8,17-BIS(4-TOLYLTHIO) INDANTHRENE	35522 	$\lambda$ 615 (3.91), 685 (4.41), 750 (4.59).

FIG. 7g.

14/28

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTHS
8,17-BIS(4-ETHOXYANILINO) INDANTHRENE	35523 	$\lambda$ 450-460 (3.96), 720 (4.40), 790 (4.53).
8,17-BIS(2-CHLOROANILINO) INDANTHRENE	35524 	$\lambda$ 455 (4.03), 710-780 (4.49), 780 (4.62).
8,17-BIS(3-CHLOROANILINO) INDANTHRENE	35525 	$\lambda$ 455 (3.81), 710-755 (4.10), 785 (4.36).

FIG. 7h.

15/28

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
8,17-BIS(4-CHLOROANILINO) INDANTHRENE	<p>35526</p>	$\lambda$ 460 (3.92), 710-715 (4.21), 785 (4.32).
8,17-BIS(4-CHLOROPHOXY) INDANTHRENE	<p>35527</p>	$\lambda$ 615 (4.21), 675 (4.45), 720-725 (4.54).
8,17-BIS(2-METHYLTHIO-ANILINO) INDANTHRENE	<p>35528</p>	$\lambda$ 460 (4.04), 710-715 (4.46), 785 (4.68).

FIG. 7i.

16/28

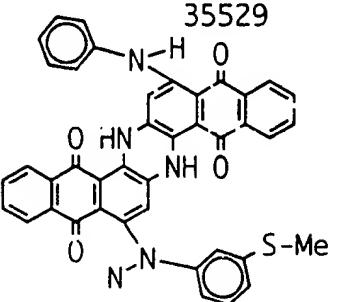
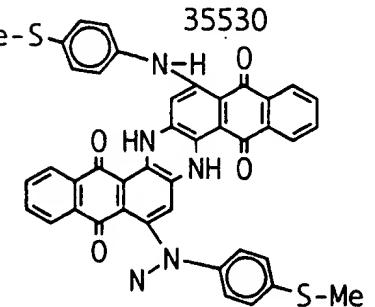
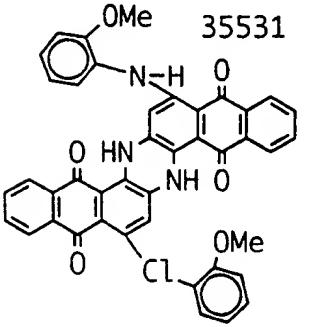
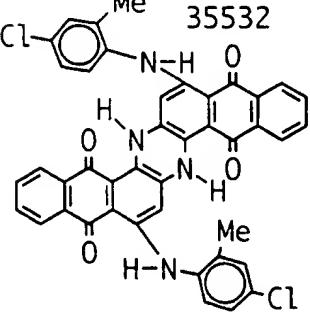
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
8,17-BIS(3-METHYLTHIO-ANILINO) INDANTHRENE		$\lambda$ 460 (418), 710-720 (4.55) 790 (4.78).
8,17-BIS(4-METHYLTHIO-ANILINO) INDANTHRENE		$\lambda$ 455 (4.15), 710-720 (4.55) 790 (4.78).
8,17-BIS(2-METHOXYPHENYL-THIO) INDANTHRENE		$\lambda$ 615 (4.00), 685 (4.48), 750 (4.61).
8,17-BIS(4-CHLORO-2-TOLUIDINO) INDANTHRENE		$\lambda$ 450 (3.96), 710-715 (4.41) 780 (4.49).

FIG. 7j.

SUBSTITUTE SHEET (RULE 26)

17/28

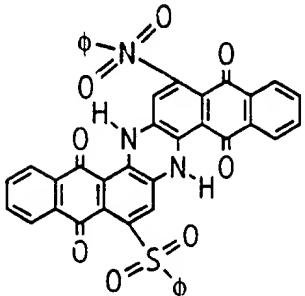
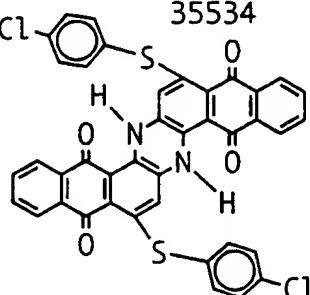
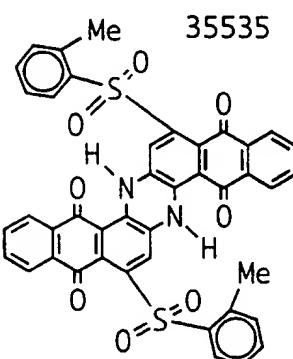
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
8,17-BIS(PHENYLSULFONYL) INDANTHRENE	35533 	$\lambda$ 610-615 (4.15), 675-685 (4.24), 700-730 (4.21).
8,17-BIS(4-CHLOROPHENYL- THIO) INDANTHRENE	35534 	$\lambda$ 615 (4.03), 685 (4.43), 750 (4.61).
8,17-BIS(2-TOLYSULFONYL) INDANTHRENE	35535 	$\lambda$ 615 (4.12), 675-685 (4.33), 705-715 (4.35).

FIG. 7k.

18/28

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
8,17-BIS(4-TOLYSULFONYL) INDANTHRENE	<p style="text-align: center;">35536</p>	$\lambda$ 615 (412), 680-690 (4.33), 705-715 (4.34).
8,17-BIS(4-BIPHENYLYL- AMINO) INDANTHRENE	<p style="text-align: center;">35537</p>	$\lambda$ 715 (4.42), 784-790 (4.52).
8,17-BIS(4-CHLOROPHENYL- SULFONYL) INDANTHRENE	<p style="text-align: center;">35538</p>	$\lambda$ 510 (4.17), 670-680 (4.24), 700-730 (4.21).

FIG. 7L.

19/28

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
36509 2-ANILINO-3,4-PHTHALOYLACRIDONE		$\lambda$ 721 (4.11)
36511 2-(4-ANISIDINO)-3,4-PHTHALOYLACRIDONE		$\lambda$ 732 (4.08)
50017 PHENYLPHENYLVINYLMETHYLUMCHLORIDE		$\lambda$ 758 (5.11)

FIG. 7m.

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
(4'-METHOXYPHENYL)-(4' METHOXY-PHENYLVINYL) METHYLUM CHLORIDE	50024 	Cl <sup>-</sup> λ 725 (5.40)
(4'-METHOXYPHENYL)-(4' METHOXY-PHENYLVINYL) METHYLUM CHLORIDE	50025 	Cl <sup>-</sup> λ 792 (5.46)
(4-DIMETHYLAMINOPHENYL)-(4' DIMETHYLLAMINOPHENYLVINYLY) METHYLUM CHLORIDE	50032 	Cl <sup>-</sup> λ 790
BINDSCHEDLER'S GREEN	52001 	Cl <sup>-</sup> λ 725
TRIS (P-DIMETHYLAMINOPHENYL) AMMENIUM PERCHLORATE	53151 	ClO4 <sup>-</sup> λ 400 (4.3), 905 (4.5)

FIG. 7n.

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
BIS (DIMETHYLAMINO) STREPTON POLYMETHINECYANINE	$(\text{Mo})_2\text{N}(\text{Me})_2 \rightleftharpoons \text{N}(\text{Me})_2 \text{Cl}^-$	$\lambda 734 (5.55)$
BIS-(4-DIMETHYLAМИНОФЕНИЛ)- СТРЕПТОПОЛИМЕТИНЦИАНИН		$\lambda 734 (5.55)$
1,1,3,3,-TETRAKIS (P-DIMETHYLAМИНОФЕНИЛ)- -2-PROPENOL PERCHLORATE z	$(\text{Me})_2\text{N}^+ \text{C}_6\text{H}_4 \text{C}_6\text{H}_3(\text{Me})_2 \text{N}(\text{Me})_2 \text{ClO}_4^-$	$\lambda 740 (4.5)$
1,1,5,5,-TETRAKIS (4-DIMETHYLAМИНО-ФЕНИЛ)- 2,4-PENTADIENOL PERCHLORATE	$(\text{Me})_2\text{N}^+ \text{C}_6\text{H}_4 \text{C}_6\text{H}_3(\text{Me})_2 \text{N}(\text{Me})_2 \text{ClO}_4^-$	$\lambda 810 (5.2)$

SUBSTITUTE SHEET (RULE 26)

FIG. 70.

22/28

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
3,3'-DIETHYL-2,2'-THIADICARBON CYANINE IODIDE		$\lambda$ 653 (5.4)
3,3'-DIETHYL-2,2'-THIATRICARBON CYANINE (IODIDE) BROMIDE [PERCHLORATE]		$\lambda$ 757, Br <sup>-</sup> , $\lambda$ 763 (5.32) (I), $\lambda$ 773 [5.32] [ClO <sub>4</sub> ]
3,3'-DIMETHYL-2,2'-THIATRICARBON CYANINE IODIDE		$\lambda$ 752 (5.38)
3,3'-DIETHYL-5,5'-DICHLORO-11-CIPHENYLAMINO-10,12-ETHYLENE-THIATRICARBOCYANINE PERCHLORATE		$\lambda$ 873 (5.19)

**SUBSTITUTE SHEET (RULE 26)**

FIG. 7p.

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
3,3'-DIETHYL-2,2'-OXATRICARBON CYANINE IODIDE		62703 I- $\lambda$ 687 (5.40)
1,1'-DIETHYL-2,2'-QUINOTRICARBON CYANINE IODIDE (CRYPTOCYANINE)		64005 I- $\lambda$ 818
1,1'-DIETHYL-4,4'-QUINOTRICARBON CYANINE IODIDE (CRYPTOCYANINE)		64102 I- $\lambda$ 710 (5.41)
1,1'-DIETHYL-11-Bromo-4,4'-QUINON DICARBOCYANINE BROMIDE		64104 Br- $\lambda$ 795
1,1',1"-TRIETHYL-11-(4'QUINOYL)-4,4'-QUINODCARBOCYANINE DIIODIDE		64106 2I- $\lambda$ 770

SUBSTITUTE SHEET (RULE 26)

FIG. 7q.

COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
64303a (C f. 51144) (64303b-ClO <sub>4</sub> <sup>-</sup> )		$\lambda$ 741 (5.38)
1,1'3,3',3'-HEXAMETHYL-2,2'-INDOTRICARBON CYANINE IODIDE (HTTC)		64304 $\lambda$ 782 (5.27)
3,3',3'-TETRAMETHYL-1,1'-BIS(4-SULFOBUTYL)-4,5,4'5'-DIBENZO-2,2'-INDOTRICARBON CYANINE, SODIUM SALT (IR-125)		64311 $\lambda$ 795 (5.29)
IR-144		64312 $\lambda$ 745 (5.09)

FIG. 7r.

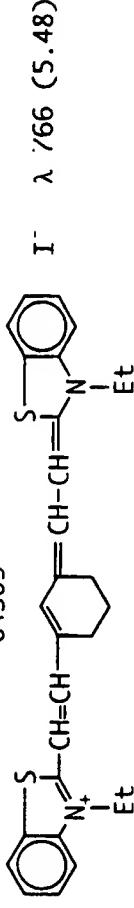
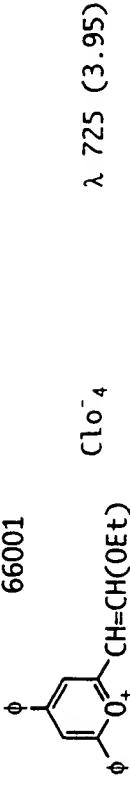
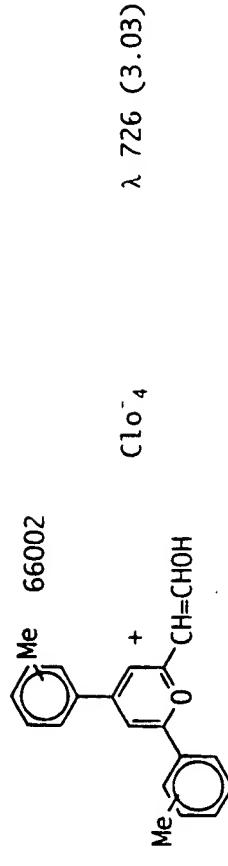
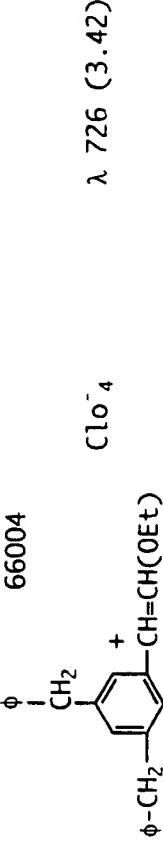
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
64303 2-(2-ETHOXYETHENYL)-4,6-DIPHENYL PYRILIUM PERCHLORATE		$\lambda = 766 \text{ (5.48)}$
66001 2-(2-HYDROXYETHENYL)-4,6-BIS(METHYLPHENYL) PYRILIUM PERCHLORATE		$\lambda = 725 \text{ (3.95)}$
66002 2-(2-ETHOXYETHENYL)-4,6-BIS(METHYLPHENYL) PYRILIUM PERCHLORATE		$\lambda = 726 \text{ (3.03)}$
66004 2-(2-ETHOXYETHENYL)-4,6-BIS(BENZYL) PYRILIUM PERCHLORATE		$\lambda = 726 \text{ (3.42)}$

FIG. 75.

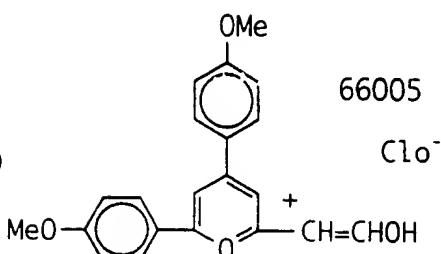
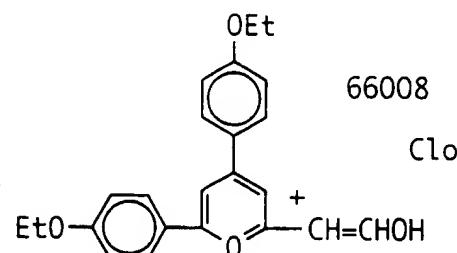
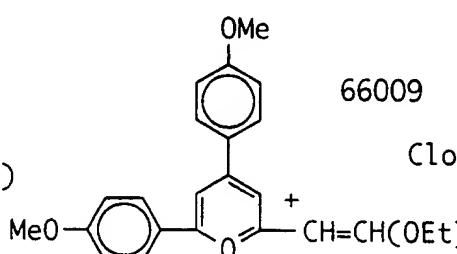
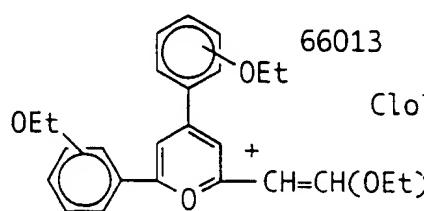
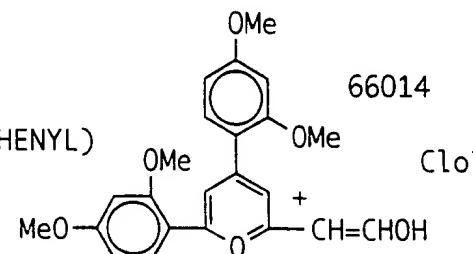
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
2-(2-HYDROXYETHENYL)- 4,6-BIS(4-METHOXYPHENYL) PYRILIUM PERCHLORATE		66005 $\text{ClO}_4^-$ $\lambda 724$ (3.54)
2-(2-HYDROXYETHENYL)- 4,6-BIS(4-ETHOXYPHENYL) PYRILIUM PERCHLORATE		66008 $\text{ClO}_4^-$ $\lambda 723$ (3.20)
2-(2-ETHOXYETHENYL)- 4,6-BIS(4-METHOXYPHENYL) PYRILIUM PERCHLORATE		66009 $\text{ClO}_4^-$ $\lambda 725$ (3.77)
2-(2-ETHOXYETHENYL)- 4,6-BIS(ETHOXYPHENYL) PYRILIUM PERCHLORATE		66013 $\text{ClO}_4^-$ $\lambda 723$ (3.60)
2-(2-HYDROXYETHENYL)- 4,6-BIS(2,4-DIMETHOXYPHENYL) PYRILIUM PERCHLORATE		66014 $\text{ClO}_4^-$ $\lambda 723$ (3.00)

FIG. 7t.

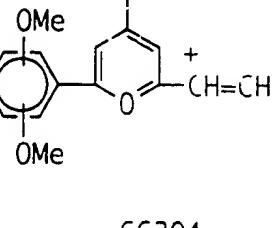
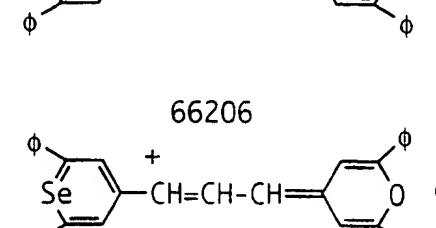
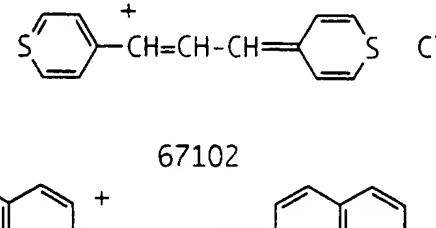
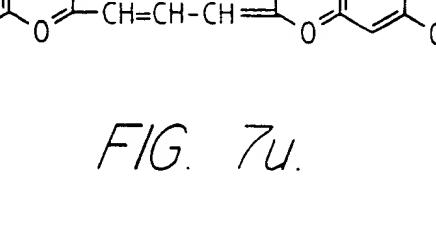
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
2-(2-ETHOXYETHENYL)-4,6-BIS(DIMETHOXYPHENYL)PYRILIUM PERCHLORATE		66002 $\text{ClO}_4^-$ $\lambda 725$ (3.01)
		66204 $\text{ClO}_4^-$ $\lambda 725$
		66206 $\text{ClO}_4^-$ $\lambda 743$
		66210 $\text{ClO}_4^-$ $\lambda 751$
		67102 $\text{ClO}_4^-$ $\lambda 745$ (4.60)

FIG. 7u.

**SUBSTITUTE SHEET (RULE 26)**

28/28

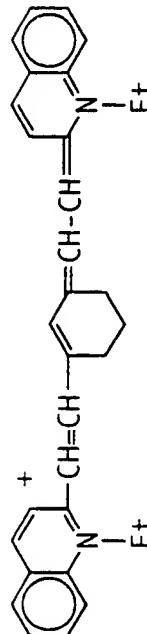
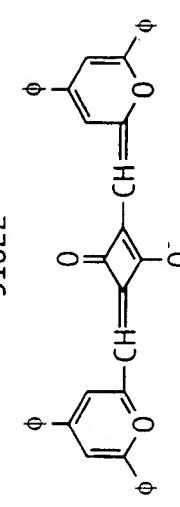
COMPOUND NAME	COMPOUND STRUCTURE	WAVELENGTH
67422		I <sup>-</sup> λ 827 (5.41)
91022		λ 815 (4.79), 910 (4.82)

FIG. 7V

**SUBSTITUTE SHEET (RULE 26)**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



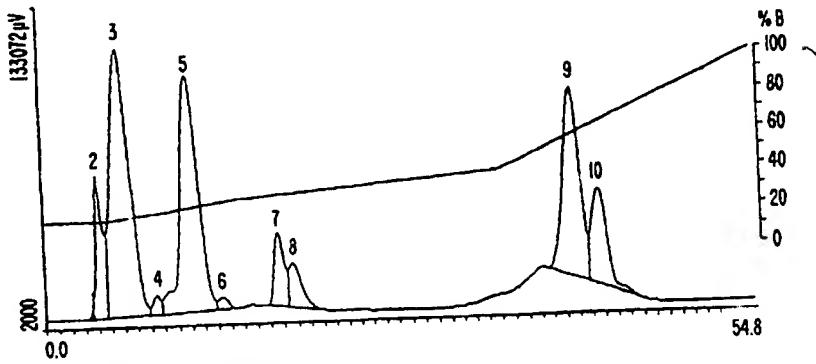
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>G01N 33/58, C09B 69/00, G01N 33/52</b>		A3	(11) International Publication Number: <b>WO 96/41144</b> (43) International Publication Date: 19 December 1996 (19.12.96)
(21) International Application Number: PCT/US96/08935 (22) International Filing Date: 4 June 1996 (04.06.96)		(81) Designated States: CA, CN, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 08/476,544 6 June 1995 (06.06.95) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant: HYPERION, INC. [US/US]; 14100 S.W. 136th Street, Miami, FL 33186 (US).		(88) Date of publication of the international search report: 6 February 1997 (06.02.97)	
(72) Inventors: DANDLICKER, Walter, Beach; 1024 Havenhurst Drive, La Jolla, CA 92037 (US). DEVLIN, Robert, Francis; 2240 Shelter Island Drive, San Diego, CA 92166 (US). ARRHENIUS, Peter, Olaf, Gustaf; 4304 Caminito del Zafiro, San Diego, CA 92121 (US). HSU, Mao-Lin; 18200 Devenwood Circle, Fountain Valley, CA 92708 (US).			
(74) Agents: BERKMAN, Charles, S. et al.; Lyon & Lyon, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).			

(54) Title: POLYOXYHYDROCARBYL MARKER PRODUCTS AND METHODS FOR FLUORESCENCE ASSAYS

(57) Abstract

Fluorescent dyes coupled to one or more polyoxyhydrocarbyl moieties which are free of aggregation and serum binding are provided. These dyes are suitable for applications such as fluorescence immunoassays, *in vivo* imaging and *in vivo* tumor therapy. Fluorescence immunoassays methods are provided which use fluorescent dyes which are free of aggregation and serum binding. Such immunoassay methods are thus, particularly useful for the assay of biological fluids, such as serum, plasma, whole blood and urine. The present invention is directed particularly to markers wherein the fluorophore is selected from the group consisting of aryl terminated polymethine dyes; quinoid dyes; 1,4-diaminoanthraquinone-2,3-dicarboxamides, indanthrene dyes; tetraaminoanthraquinones; azine dyes; (thio)pyriliandyes and naphthoquinone methides.



ANALYSIS: CHANNEL A

PEAK NO.	TIME	TYPE	HEIGHT(μV)	AREA(μV-sec)	AREA %
1	3.468	N1	3483	22063	0.061
2	4.051	N2	4.051	2268025	6.290
3	5.885	N3	58612	11074450	30.717
4	8.375	N4	110393	313179	0.868
5	11.071	N5	8152	9470101	26.267
6	13.351	N6	96740	26815	0.731
7	17.631	N1	5092	1504825	4.173
8	18.710	N2	17949	1199819	3.327
9	40.655	N1	76569	6775683	18.793
10	42.630	N2	38484	3160570	8.766
TOTAL AREA				36052530	99.993

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Larvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

# INTERNATIONAL SEARCH REPORT

International Application No  
NL/US 96/08935

**A. CLASSIFICATION OF SUBJECT MATTER**  
**IPC 6 G01N33/58 C09B69/00 G01N33/52**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
**IPC 6 G01N C09B**

Documentation searched other than minimum documentation, to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 19366 A (DIATRON CORP) 30 September 1993	1-16
Y	see page 36 - page 37	17-20, 35-37
X	---	1-16
Y	WO 91 18007 A (DIATRON CORP) 28 November 1991 see page 10 - page 12; claim 1	17-20, 35-37,39
X	---	1-16
Y	WO 91 18006 A (DIATRON CORP) 28 November 1991 see page 5; claim 6	17-20, 35-37,39
Y	---	17-20, 35-37,39
Y	US 5 378 634 A (SIGETOH NOBUYUKI ET AL) 3 January 1995 see the whole document	17-20, 35-37,39
	---	- / -

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- '&' document member of the same patent family

1

Date of the actual completion of the international search

Date of mailing of the international search report

10 October 1996

03.01.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl.  
Fax (+ 31-70) 340-3016

Authorized officer

Wells, A

# INTERNATIONAL JOURNAL OF RESEARCH REPORT

**International Application No.**

PC., US 96/08935

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 609 894 A (CANON KK) 10 August 1994	17-20,
	see the whole document	35-37,39
---		
X	CLINICAL CHEMISTRY, vol. 39, 1993, pages 1939-1943, XP002015528	1-16
	R. DEVLIN ET AL: "Homogeneous Detection of Nucleic Acids by Transient-State Polarized fluorescence"	
Y	see the whole document	17-20,
	-----	35-37,39
-----		

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/08935

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See for further information appended sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searching claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-16 (in part),  
17-20, 35-37, 39 (complete)

### Remark on Protest

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International Application No. PCT/US 96/ 08935

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/210**

The inventions identified are the following:

- (i) Claims 1-16(part), 17-20, 35 36, 37, 39; polymethine markers
- (ii) Claims 1-16(part), 21-23; quinoid markers
- (iii) Claims 1-16(part), 24, 32, 33, 34; indanthrene markers
- (iv) Claims 1-16(part), 25; anthraquinone dicarboxamide markers
- (v) Claims 1-16(part), 26; tetraaminoanthraquinone markers
- (vi) Claims 1-16(part), 27; azine markers
- (vii) Claims 1-16(part), 28, 38; (thio)pyrilium markers
- (viii) Claims 1-16(part), 29,31; napthoquinone methide markers
- (ix) Claims 1-16(part), 30; carbazine markers

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/08935

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9319366	30-09-93	CA-A- EP-A-	2132708 0632893	30-09-93 11-01-95
WO-A-9118007	28-11-91	CA-A- EP-A-	2082934 0528991	16-11-91 03-03-93
WO-A-9118006	28-11-91	CA-A- EP-A- US-A-	2082936 0529002 5403928	16-11-91 03-03-93 04-04-95
US-A-5378634	03-01-95	JP-A- JP-A- US-A-	6066725 6157926 5488114	11-03-94 07-06-94 30-01-96
EP-A-0609894	10-08-94	JP-A-	6230007	19-08-94